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Serial No.: 09/382,622

Filed: August 25, 1999

For: High Energy Phototherapeutics Agents

Examiner: G. Gabel

Art Unit: 1641

APPELLANTS' BRIEF UNDER 37 C.F.R. 1.192

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Groenwald et al., A Clinical Guide to Cancer Nursing, p. 61-62 (1995) H

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Neckers, Journal of Photochemistry and Photobiology, A: Chemistry (1989) L

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APPELLANT'S BRIEF UNDER 37 C.F.R. §1.192

This Brief is in furtherance of the Notice of Appeal filed in this Application Serial No. 09/382,622 on March 12, 2002.

This application is a divisional of USSN 09/216,787 filed on December 21, 1998 (now U.S. Patent 6,331,286).

A first Office Action (Exhibit A) in this divisional application issued August 29, 2000. In that Office Action, the Examiner objected to informalities in the specification and stated the following rejections of the claims:

- Claims 2-30 and 52 under 35 U.S.C. §112, second paragraph as being indefinite for informalities in the claims.
- Claims 1-30, 51 and 52 under 35 USC §112, first paragraph, as being enabling only for treatment of diseased tissue such as cancer and tumor tissue but not enabling for any diseased tissue.
- Claims 1-3, 5-8, 10, 12-13, 15-16, 23, 28-30 and 51 under 35 USC §102 as being anticipated by Serafini et al. (Journal of Nuclear Medicine, 1975) (Exhibit K).
- Claims 1-3, 5-10, 12-13, 15-16, 23, 28-30 and 51 under 35 USC §102 as being anticipated by Neckers (Journal of Photochemistry and Photobiology, A: Chemistry 47: 1-29 (1989)) (Exhibit L).
- Claims 12-17, 21-24, 28-29 and 52 under 35 USC §102 as being anticipated by Norman et al. (Invest Radiol, 26: S120-S121 (1991)) (Exhibit N).
- Claims 18 and 25 under 35 USC §103 as not being patentable over Norman et al. in view of Khaw et al. (US 5,780,052) (Exhibit M).

- Claims 4, 11, 14, 17-22 and 24-27 under 35 USC §103 as not being patentable over Serafini et al. or Neckers in view of Khaw et al.

Thus, no claims were allowed.

Appellants gave extensive consideration to the Office Action, and in Amendment A (11-29-00), Appellants amended the claims and specification to overcome the objected to informalities. Appellants then explained why the claims are definite, are enabling and are patentable over the cited references.

The Examiner then issued a Final Rejection on February 27, 2001 (Exhibit B). In the Final Rejection, the Examiner withdrew some of the §112, second paragraph indefiniteness rejections but continued to reject Claims 15-16, 23, and 25-27 thereunder. The Examiner further withdrew the following rejections:

- The §112, first paragraph rejection for enablement.
- Claims 10, 15-16, 23 and 51 under 35 USC §102 as being anticipated by Serafini et al.
- Claims 10, 15-16, 23 and 51 under 35 USC §102 as being anticipated by Neckers;
- Claims 12-16, 23, and 52 under 35 USC §102 as being anticipated by Norman et al.
- Claims 18 and 25 over Norman et al. under 35 USC §103 as being unpatentable in view of Khaw et al..

The Examiner, however, maintained the following rejections:

- Claims 1-3, 5-8 and 12-13, plus new Claims 58-59 under 35 USC §102 as being anticipated by Serafini et al.
- Claims 1-3, 5-9 and 12-13, plus new Claims 58-59 under 35 USC §102 as being anticipated by Neckers.

- Claims 4, 14, 18-20 and 25-27 under 35 USC §103 as being unpatentable over Serafini or Neckers et al. in view of Khaw.

The Examiner added the following new rejections:

- Claims 15-16, 23 and 54-55 under 35 USC §103 as being unpatentable over Serafini et al. or Neckers in view of Khaw et al.
- Claims 10, 51, 52 and 56-57 under 35 USC §103 as being unpatentable over Serafini et al. or Neckers in view of Norman et al.

Thus, no claims were allowed.

Thereafter, counsel for Appellants had a telephonic interview with the Examiner on May 10, 2001, based on a proposed draft Amendment faxed to the Examiner. On May 25, 2001, Appellants submitted Proposed Amendment B (After Final) with amendments based on the Examiner's comments during the interview.

Much to the Appellants' surprise and contrary to their conversation during the May 10, 2001 interview, the Examiner left a voice mail message for counsel stating that the amendment would not be entered due to allegedly new issues and unsupported new recitations incorporated into the claims. See 6-18-01 Interview Summary (Exhibit C).

Appellants then submitted on June 27, 2001 a new Amendment B (After Final), with an extension of time petition.

The Examiner then issued an Advisory Action dated July 25, 2001 (Exhibit D) and refused to enter the amendment, deciding that the amendment did not place the application in a condition for allowance but raised new matter and new consideration issues. The Examiner further stated that she would not enter the amendment because she alleged that the amendment to Claim 1 introduced

new §112 first and second paragraph issues in reciting "halogenated xanthene interacting with ionizing radiation...to enhance therapeutic efficacy of ionizing radiation."

Appellants filed a RCE on August 21, 2001 (with a petition to extend time) to consider the amendment that was not previously entered.

The Examiner then issued yet another Office Action on September 13, 2001 (Exhibit E). In that Office Action, the Examiner withdrew the rejection of Claims 15-16, 23, 25-27 and 54 under 35 U.S.C §112. The Examiner also withdrew the rejection of Claims 25-27 under 35 U.S.C §103 as being unpatentable over Serafini, et al. or Neckers in view of Khaw, et al. The Examiner, however, maintained the following rejections:

- Claims 1-3, 5-8 and 12-13 under 35 U.S.C §102 as being anticipated by Serafini, et al.
- Claims 1-3, 5-9 and 12-13 under 35 U.S.C §102 as being anticipated by Neckers.
- Claims 4, 14 and 18-20 under 35 U.S.C §103 as being unpatentable over Serafini, et al. or Neckers in view of Khaw, et al.
- Claims 10, 51, 52 and 56-57 as being unpatentable over Serafini, et al. or Neckers in view of Norman, et al.

Thus, no claims were allowed.

The Examiner also had specific comments on pages 4-10 of the Office Action. In general, the Examiner's comments state that while none of the references disclose use of a halogenated xanthene as a radiosensitizer agent for use with ionizing radiation to treat cancer or tumors and while each discloses a use completely different from that of Claim 1, the references disclose the same structure Rose Bengal as used in the present application, and therefore the claims would be anticipated or obvious over these references.

Appellants then filed the Notice of Appeal, along with Amendment C (Exhibit F) adding a few dependent claims.

The claims of the present application are clearly patentable over the cited references, as will be shown *infra*, and Appellants respectfully request the Board to so rule and allow the application.

I. STATEMENT OF REAL PARTY IN INTEREST

The real party in interest in this appeal is the assignee: Photogen, Inc., 7327 Oak Ridge Highway, Knoxville, TN 37931.

II. STATEMENT OF RELATED APPEALS AND INTERFERENCES

To the best of Appellants' and Appellants' legal representative's knowledge, there are no appeals or interferences pending which will affect or be affected by the Board's decision in this appeal.

III. STATUS OF CLAIMS

The rejection of Claims 1-10, 14, 15, 18-20, 50-52 and 55-57 of the appealed claims appears in Section IX, *infra* at p. 43 *et seq.* Also pending are new (dependent) Claims 60-67 in this application.

IV. STATUS OF AMENDMENTS

Amendment C (Exhibit F) is currently outstanding in this application.

V. SUMMARY OF INVENTION

The present invention, as recited in independent Claims 1, 10, and 51, concerns a novel radiosensitizer agent for treatment of cancer and tumors. The radiosensitizer agent comprises a halogenated xanthene that interacts with ionizing radiation to enhance the therapeutic efficacy of the radiation, or is activated using x-rays.

The specification of the present application provides support and explanation for the features of independent Claims 1, 10, and 51, as described below.

More specifically, Claim 1 is directed to a radiosensitizer agent for treatment of cancer and tumors. The radiosensitizer agent comprises a halogenated xanthene. The halogenated xanthene interacts with ionizing radiation applied to the cancer or tumor to enhance the therapeutic efficacy of the ionizing radiation. The features of this claim are shown in the specification, for example, at page 5, lns. 2-4; page 6, lns. 10-20; page 10, lns. 10-12.

Claims 2-9, 14, 15, 18-20, 52 and 55-57 are dependent, either directly or indirectly, on Claim 1, and accordingly, all the elements listed above for Claim 1 are included within Claims 2-9, 14, 15, 18-20, 52 and 55-57 and the support for these elements in the specification is the same as mentioned *supra*. These dependent claims also add additional elements or limitations which are supported in the specification as follows:

Claim 2 is dependent on Claim 1 and specifies that the halogenated xanthene comprises Rose Bengal. Page 5, lns. 2-4; page 8, lns. 19-20; page 10, lns. 4-9, 13-15; Table 1.

Claim 3 is dependent on Claim 1 and specifies that the halogenated xanthene comprises 4,5,6,7-Tetrabromoerythrosin. Page 10, lns. 4-9, 13-15; Table 1.

Claim 4 is dependent on Claim 1 and specifies that the halogenated xanthene includes as a functional derivative at least one targeting moiety selected from the group consisting of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), amino acids, proteins, antibodies, ligands, haptens, carbohydrate receptors or complexing agents, lipid receptors or complexing agents, protein receptors or complexing agents, chelators, encapsulating vehicles short- or long-chain aliphatic or aromatic hydrocarbons, aldehydes, ketones, alcohols, esters, amides, amines, nitriles, and azides. Page 9, lns. 3-16.

Claim 5 is dependent on Claim 1 and specifies that the halogenated xanthene also is an imaging contrast agent. Page 5, lns. 5-6; page 10, lns. 19-20.

Claim 6 is dependent on Claim 5 and specifies that the halogenated xanthene acts as an imaging contrast agent for computed axial tomography. Page 10, lns. 19-20.

Claim 7 is dependent on Claim 5 and specifies that the halogenated xanthene acts as an imaging contrast agent for X-ray imaging. Page 10, lns. 19-20.

Claim 8 is dependent on Claim 1 and specifies that the halogenated xanthene includes at least one halogen selected from the group consisting of iodine and bromine. Page 10, ln. 1.

Claim 9 is dependent on Claim 1 and specifies that the halogenated xanthene is selected from the group consisting of Phloxine B, Erythrosin B and Eosin Y. Page 10, lns. 4-9; Table 1.

Claim 14 is dependent on Claim 1 and specifies that wherein at least one biological targeting moiety is attached to the halogenated xanthene to enhance targeting of said halogenated xanthene to biologically sensitive structures of said cancer or tumors. Page 5, lns. 7-11; page 7, lns. 1-2; page 9, lns. 3-13.

Claim 15 is dependent on Claim 1 and specifies that at least one chemical targeting moiety is attached to the halogenated xanthene to enhance targeting of said halogenated xanthene to biologically sensitive structures of said cancer or tumors. Page 5, lns. 10-11; page 7, lns. 1-2; page 9, lns. 3-13.

Claim 18 is dependent on Claim 1 and specifies that the halogenated xanthene is encapsulated in a delivery vehicle. Page 7, lns. 18-21.

Claim 19 is dependent on Claim 18 and specifies that the halogenated xanthene is Rose Bengal. Page 5, lns. 3-4; page 8, lns. 19-20.

Claim 20 is dependent on Claim 18 and specifies that the delivery vehicle is selected from the group consisting of micelles, nanoparticles and liposomes. Page 7, lns. 18-21.

Claim 52 is dependent on Claim 1 and specifies that the ionizing radiation is approximately greater than or equal to 1 keV and less than or equal to approximately 1000 MeV. Page 10, lns. 15-17.

Claim 55 is dependent on Claim 1 and specifies that the halogenated xanthene includes as a functional derivative at least one targeting moiety selected from the group consisting of hydrophilic and hydrophobic moieties. Page 9, lns. 12-13.

Claim 56 is dependent on Claim 1 and specifies that the ionizing radiation comprises x-rays. Page 6, lns. 10; page 10, lns. 15-16.

Claim 57 is dependent on Claim 56 and specifies that the x-rays have an energy between 30 kiloelectron volts and 1000 megaelectron volts. Page 10, lns. 15-18.

Claim 10 is a further independent claim directed to a radiosensitizer agent for treatment of cancer and tumors using ionizing radiation. The radiosensitizer agent comprises a halogenated

xanthene. The halogenated xanthene is activated using x-rays having an energy greater than 30 keV. These features are shown in the specification, for example, at page 5, lns. 2-4; page 6, lns. 10-20; page 10, lns. 1-12, 17-18.

Claims 60-63 are dependent, either directly or indirectly, on Claim 10, and accordingly, all the elements listed above for Claim 10 are included within Claims 60-63 and the support for these elements in the specification is the same as mentioned *supra*. These dependent claims also add additional elements or limitations which are supported in the specification as follows:

Claim 60 is dependent on Claim 10 and specifies that the halogenated xanthene comprises Rose Bengal. Page 5, lns. 2-4; page 8, lns. 19-20; page 10, lns. 4-9, 13-15; Table 1.

Claim 61 is dependent on Claim 10 and specifies that the halogenated xanthene includes as a functional derivative at least one targeting moiety selected from the group consisting of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), amino acids, proteins, antibodies, ligands, haptens, carbohydrate receptors or complexing agents, lipid receptors or complexing agents, protein receptors or complexing agents, chelators, encapsulating vehicles short- or long-chain aliphatic or aromatic hydrocarbons, aldehydes, ketones, alcohols, esters, amides, amines, nitriles, and azides. Page 9, lns. 3-16.

Claim 62 is dependent on Claim 10 and specifies that the halogenated xanthene also is an imaging contrast agent. Page 10, lns. 19-20.

Claim 63 is dependent on Claim 10 and specifies that the halogenated xanthene is encapsulated in a delivery vehicle. Page 7, lns. 18-21.

Claim 51 is a further independent claim directed to a radiosensitizer agent for treatment of cancer and tumors using radiosensitization or ionizing radiation. The radiosensitizer agent comprises

a halogenated xanthene, and the ionizing radiation is approximately greater than or equal to 1 keV and less than or equal to approximately 1000 MeV. These features are shown in the specification, for example, at page 5, lns. 2-4; page 6, lns. 10-20; page 10, lns. 10-12, 15-17.

Claims 64-67 are dependent, either directly or indirectly, on Claim 51, and accordingly, all the elements listed above for Claim 51 are included within Claims 64-67 and the support for these elements in the specification is the same as mentioned *supra*. These dependent claims also add additional elements or limitations which are supported in the specification as follows:

Claim 64 is dependent on Claim 51 and specifies that the halogenated xanthene comprises Rose Bengal. Page 5, lns. 2-4; page 8, lns. 19-20; page 10, lns. 4-9; Table 1.

Claim 65 is dependent on Claim 51 and specifies that the halogenated xanthene includes as a functional derivative at least one targeting moiety selected from the group consisting of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), amino acids, proteins, antibodies, ligands, haptens, carbohydrate receptors or complexing agents, lipid receptors or complexing agents, protein receptors or complexing agents, chelators, encapsulating vehicles short- or long-chain aliphatic or aromatic hydrocarbons, aldehydes, ketones, alcohols, esters, amides, amines, nitriles, and azides. Page 9, lns. 3-16.

Claim 66 is dependent on Claim 51 and specifies that the halogenated xanthene also is an imaging contrast agent. Page 10, lns. 19-20.

Claim 67 is dependent on Claim 51 and specifies that the halogenated xanthene is encapsulated in a delivery vehicle. Page 7, lns. 18-21.

VI. ISSUES

1. Whether Claims 1-3 and 5-8 are anticipated under 35 U.S.C §102 by Serafini, et al.?

2. Whether Claims 1-3 and 5-9 are anticipated under 35 U.S.C §102 by Neckers?

3. Whether Claims 4, 14 and 18-20 would have been obvious under 35 U.S.C §103 over Serafini, et al. or Neckers in view of Khaw, et al.?

4. Whether Claims 10, 51, 52 and 56-57 would have been obvious under 35 U.S.C §103 over Serafini, et al. or Neckers in view of Norman, et al.?

VII. GROUPING OF CLAIMS

It is submitted that the claims do not stand or fall together but are separately patentable. Claims 1, 10 and 51 are independent claims and are directed to different aspects of the present invention or are in varying detail. They are separately argued. The dependent claims have additional patentable features, and many are separately argued.

Further reasons for the separate patentability of the claims are included within the Argument (see Section VIII, *infra*).

VIII. ARGUMENT

A. BACKGROUND

The present invention is directed to radiosensitizer agents. As explained in depth *infra*, radiosensitizer agents are medicinal compositions that will be used with ionizing radiation to treat cancers and tumors. The present invention concerns a novel radiosensitizer agent comprising a halogenated xanthene. The claims are directed to various aspects of this radiosensitizer agent

including compositions that interact with ionizing radiation or compositions that are activated by x-rays for treatment of cancers and tumors.

The inventors of the present application have made a remarkable new discovery -- that a radiosensitizer agent comprising Rose Bengal or other halogenated xanthenes interacts with ionizing radiation or is activated by x-rays to enhance the efficacy of such radiation in treating cancer and tumors. Appellants have also discovered combinations claimed herein, but this radiosensitizer agent of the present invention has heretofore been completely unknown, so far as can be determined (as the Examiner appears to agree).

This radiosensitizer agent represents a significant breakthrough in cancer treatment. The inventors have discovered that the halogenated xanthenes exhibit intrinsic targeting and concentration in diseased tissue, such as cancerous and tumor tissue. Once there, the halogenated xanthenes interact with applied ionizing radiation, such as x-rays, or are activated by the radiation, to enhance the therapeutic efficiency of the radiation, thereby producing a superior cancer tumor fighting agent that efficiently kills the cancer or tumor cells.

Further, the halogenated xanthenes have been shown to be non-toxic (except in the very high concentration in tumors and cancer cells of the Photogen inventions) and have a relative low cost. The result is an effective, cost-efficient cancer/tumor fighting agent that is safe, kills cancer/tumor cells and has little or no effect on the healthy cells in the surrounding tissue.

In contrast, the primary references cited by the Examiner in the Final Rejection are general disclosures regarding certain chemical and physical properties of Rose Bengal, which is one halogenated xanthene. The claims of the present application, however, are not directed to claiming merely Rose Bengal. Instead, the claims of the present application are very specifically directed to

a radiosensitizer agent which comprises a halogenated xanthene which interacts with or is activated by ionizing radiation. As explained below, a radiosensitizer agent is a very specific class of medicinal agent for use with ionizing radiation to treat cancers and tumors. None of the cited references disclose the radiosensitizer agent of the present invention.

Radiosensitizers/Radiosensitizer Agents

The terms "radiosensitizer" and "radiosensitizer agent" are used throughout the specification of the present application. Radiosensitizer agents are medicinal compositions which interact with ionizing radiation or are activated by the ionizing radiation to enhance the therapeutic efficiency of the radiation to treat cancer and tumors. For example, the specification discusses "radiosensitizer" and "radiosensitizer agents" early in the application:

> "Others, however, have focused their efforts on developing agents that are sensitized or activated by the ionizing radiation mentioned above. Potentially, the use of such radiation would enable treatment of more deeply seated diseased tissue than that possible with optical radiation. The agents used with such radiation are known as radiosensitizers. It is also desirable to achieve preferential concentration of the radiosensitizer in the diseased tissue, either through natural processes or via localized application, so as to provide additional specificity relative to that achievable through standard radiation therapy. The desired result is for radiation to become more efficacious when the radiosensitizer is present in tissue, so that less radiation is needed to treat the lesion tumor or other diseased tissue, and, accordingly, potential damage to surrounding healthy tissue, resulting from collateral exposure to the radiation, is reduced."

Specification, page 2, lines 17-20 (emphasis added). Other citations occur on nearly every page thereafter in the specification. Such as for example, on page 9, lns. 22-24:

> "In fact, the halogen content of the halogenated xanthenes makes this class of agent potent x-ray absorbers, and thus highly suitable as radiosensitizers."

An on-line dictionary (Biotech Life Science Dictionary (1995-1998)) defines "radiosensitizer" as follows:

> "A radiosensitizer is a substance given to a patient to make the intended target (such as a tumor) more susceptible to the effects of radiotherapy [i.e. ionizing radiation]." (Exhibit G)

Further, Groenwald et al., A Clinical Guide to Cancer Nursing, p. 61-62 (1995) (Exhibit H) states that radiosensitizers are in the class of "Chemical and thermal modifiers of radiation" and that such radiosensitizers "increase radiosensitivity of tumor cells" and "achieve greater cell kill." Groenwald et al. expanded on that in 1997 in Cancer Nursing Principles and Practice, 4th ed., p. 260-261 (1997) (Exhibit I):

> "The goal of radiotherapy is to achieve maximum tumor cell kill while minimizing injury to normal tissues (therapeutic ratio). Efforts to improve the therapeutic ratio have resulted in development of certain compounds that act to increase the radiosensitivity of tumor cells...Combined modality therapy with both radiation and certain cytotoxic agents also takes advantage of enhanced tumor cell kill. Drugs...often are used along with radiation to achieve greater cell kill than either therapy could achieve if used independently. When used alone, chemical modifiers of radiation therapy (radiosensitizers), however, are not generally cytotoxic like the chemotherapeutic agents."

As explained in depth *infra*, none of the primary cited references in the Office Action of September 13, 2001 discloses or suggests a radiosensitizer agent, and none of the references discloses or suggest a radiosensitizer agent comprising a halogenated xanthene, in general, or Rose Bengal, in particular, is a radiosensitizer or radiosensitizer agent for interacting with or activation by ionizing radiation.

Also, as indicated by the above citations, it is clear to those skilled in the art that the radiosensitizers or radiosensitizer agents described in the present invention are medicinal compositions that are administered to patients as an adjuvant with ionizing radiation in order to improve the efficacy of radiation therapy.

FDA Regulations

As will be shown, granting a patent containing the appealed claims will not restrict normal commerce in Rose Bengal or other halogenated xanthenes outside the field of radiosensitizers agents for treatment of cancers and tumors with ionizing radiation.

In particular, the U.S. Food and Drug Administration ("FDA") carefully regulates interstate commerce in all such agents or medicinal compositions/drugs approved for human use in the U.S. The FDA has strict labeling requirements (Exhibit J) concerning identification of the manufacturer, directions for use, ingredients, and intended use for any drug sold in interstate commerce. For example, concerning identification of manufacturer, 21 CFR §201.1 ("Drugs; name and place of business of manufacturer, packer, or distributor") states:

> "(a) A drug or drug product (as defined in Sec. 320.1 of this chapter) in finished package form is misbranded under section 502 (a) and (b)(1) of the act if its label does not bear conspicuously the name and place of business of the manufacturer, packer, or distributor..."

Further, 21 CFR §201.5 ("Drugs; adequate directions for use") requires detailed labeling concerning drug usage and dosage:

> "*Adequate directions for use* means directions under which the layman can use a drug safely and for the purposes for which it is intended. (Section 201.128 defines "intended use.") Directions for use may be inadequate because, among other reasons, of omission, in whole or in part, or incorrect specification of:

(a) Statements of all conditions, purposes, or uses for which such drug is intended, including conditions, purposes, or uses for which it is prescribed, recommended, or suggested in its oral, written, printed, or graphic advertising, and conditions, purposes, or uses for which the drug is commonly used; except that such statements shall not refer to conditions, uses, or purposes for which the drug can be safely used only under the supervision of a practitioner licensed by law and for which it is advertised solely to such practitioner.

(b) Quantity of dose, including usual quantities for each of the uses for which it is intended and usual quantities for persons of different ages and different physical conditions.

(c) Frequency of administration or application.

(d) Duration of administration or application.

(e) Time of administration or application (in relation to time of meals, time of onset of symptoms, or other time factors).

(f) Route or method of administration or application.

(g) Preparation for use, i.e., shaking, dilution, adjustment of temperature, or, other manipulation or process." (Emphasis in original)

Concerning quantitative, definitive identification of drug ingredients, 21 CFR §201.10 ("Drugs; statement of ingredients") states:

"(a) The ingredient information required by section 502(e) of the Federal Food, Drug, and Cosmetic Act shall appear together, without any intervening written, printed, or graphic matter, ...

(b) The term *ingredient* applies to any substance in the drug, whether added to the formulation as a single substance or in admixture with other substances..." (Emphasis in original)

Finally, concerning intended use (i.e., indications) for a drug, 21 CFR §201.128 ("Meaning of 'intended uses' ") states:

"The words *intended uses* or words of similar import in Secs. 201.5, 201.115, 201.117, 201.119, 201.120, and 201.122 refer to the objective intent of the persons legally responsible for the labeling of drugs. The intent is determined by such persons' expressions or may be shown by the circumstances surrounding the distribution of the article. This objective intent may, for example, be shown by labeling claims, advertising matter, or oral or written statements by such persons or their representatives." (Emphasis in original)

Therefore, any drug product, such as a radiosensitizer agent, will, within all jurisdictions of the U.S. (i.e., all jurisdictions falling under the auspices of the U.S. FDA, as well as those of the U.S. PTO), be strictly regulated by the FDA, and will require detailed labeling concerning manufacturer, composition and intended use. As such, the commercial non-drug channels for a reagent grade material (for example reagent grade Rose Bengal from a chemical supply house, such as for example Sigma Chemical, used for laboratory, industrial or cosmetic purposes) will be completely distinct from those for any drug or drug product (for example a radiosensitizer agent approved for use in the U.S., which must comply with FDA regulations concerning drugs and drug products) and will not be permitted to be sold or used as a radiosensitizer or combined therapeutically with ionizing radiation. Likewise, a drug for diagnostic purposes and which is not for interaction with ionizing radiation (such as that described e.g. by Serafini et al. *infra*) would also be completely distinct from a radiosensitizer agent.

Accordingly, the granting of Appellants' claims, such as for example independent Claim 1, for a radiosensitizer agent for treatment of cancer and tumors, said radiosensitizer agent comprising a halogenated xanthene which interacts with ionizing radiation applied to said cancer or tumor to enhance the therapeutic efficacy of said ionizing radiation, will in no way impact commercial trade in non-drug compositions of the halogenated xanthenes, nor will it affect any other *non-radiosensitizer medicinal use* of the halogenated xanthenes[1] which are not combined therapeutically with ionizing radiation.

[1] For example, as described *infra* for Serafini et al., use of radiolabelled Rose Bengal for diagnosis of liver disease, which will clearly require very different labeling concerning intended use, will be unaffected.

The Claims

The present claims are not directed merely to halogenated xanthenes, and Appellants concede that halogenated xanthenes are known substances. That has not been contested by Appellants. Instead, the claims are directed to a specific class of medicinal compositions, i.e. radiosensitizer agents, and novel and non-obviousness combinations, which include halogenated xanthenes and ionizing radiation for specific medicinal purposes.

For example, independent Claim 1 is for:

- a radiosensitizer agent for treatment of cancers and tumors;
- a radiosensitizer agent comprising a halogenated xanthene;
- the halogenated xanthene interacting with ionizing radiation applied to a cancer or tumor to enhance the therapeutic efficiency of the ionizing radiation.

This claim therefore is not promulgated with the objective of prohibiting all commerce in halogenated xanthenes, but only to obtain the patent right to exclude radiosensitizer agents which comprise halogenated xanthenes which interact with ionizing radiation for application to cancers and tumors, for enhancing the therapeutic efficiency of ionizing radiation. As explained above, commerce in medicinal compositions (or substances) to be combined with ionizing radiation for cancer treatment is strictly regulated by the FDA, and as a result, specific pharmaceutical, drug or other medicinal products are easily distinguishable, commercial entities, even if they have some comparable active ingredients.

Similarly, independent Claim 10 is directed to:

- a radiosensitizer agent for treatment of cancer or tumors;
- a radiosensitizer agent comprising a halogenated xanthene;

- the halogenated xanthene being activated using x-rays having an energy greater than 30 keV.

As a result, the subject matter of this claim falls into the same class, and its commerce would be similarly strictly regulated. The other pending claims would also fall into the same class.

The PTO has recognized the patentability of methods using halogenated xanthenes that are disclosed in the instant application and has granted U.S. Patent No. 6,331,286 for Appellants' new and nonobvious methods. Appellants assert in this divisional application that they are entitled to patent rights for the radiosensitizer agents comprising halogenated xanthenes for treatment of cancer and tumors with ionizing radiation.

As a result and as explained in more depth below, Appellants respectfully submit that the claims of the present application are clearly distinguishable and patentable over the cited references and should now be allowed.

The rejections are now addressed in the order in which they are given in the Office Action of September 13, 2001 (Exhibit E).

B. CLAIMS 1-3 AND 5-8 ARE NOT ANTICIPATED BY SERAFINI ET AL.

In the Office Action, the Examiner rejected Claims 1-3, 5-8 and 12-13[2] under 35 USC §102(b) as being "inherently anticipated" by Serafini et al.

This rejection is erroneous as the claimed invention and Serafini et al. are very different. Further, this reference does not disclose or suggest all of the elements of the rejected claims, as required for an anticipation rejection.

[2]Claims 12 and 13 were canceled in Amendment B, and the rejection of these claims is moot.

1. Anticipation Requires Every Element To Be Found In A Single Reference

Anticipation under 35 USC §102 is a very technical rejection. In order to prove anticipation, it must be shown that every single element of a claim is found in a single prior art reference. Union Oil Company of California v. Atlantic Richfield Co., 208 F.3d 989, 994-995; 54 USPQ2d 1227, 1231-1232 (Fed. Cir. 2000); Kloster Speed Steel AB v. Crucible Inc., 793 F.2d 1565, 1571, 230 USPQ 81, 84 (Fed.Cir. 1986); Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1379, 231 USPQ 81, 90 (Fed. Cir. 1986). It is not enough that a prior art reference discloses some aspects of a claim; all of the elements must be disclosed in a single reference. Absence from the prior art reference of any claimed element negates a finding of anticipation. Kloster Speed Steel, 793 F.2d at 1571, 230 USPQ at 84; *see also* Atlas Powder Co. v. E.I. DuPont, 750 F.2d 1569 (Fed. Cir. 1984).

2. Serafini et al.

Serafini et al. (Exhibit K) describes the use of certain highly radioactive forms of Rose Bengal in the medical evaluation of liver function.[3] In a *diagnostic* test, a radioactive form of the compound (radiolabelled with iodine-131 or, as taught by Serafini et al., with iodine-123) is administered to the patient as an intravenous bolus. The subsequent distribution of the diagnostic agent throughout certain organs of the patient's body is then determined *by detecting the emissions* resulting from the spontaneous radioactive decay of the radioactive iodine contained in this radiolabelled Rose Bengal. Serafini et al. uses a radiation detection system (i.e., a "scintillation camera") that is placed outside the patient's body, for detecting this spontaneous radioactive decay

[3] Appellants note that the compounds used in radiosensitizer agents, such as the halogenated xanthenes claimed in the present application are typically non-radioactive.

of the radioactive iodine. (The scintillation camera receives and produces an electrical or other signal based on the detected radiation). Serafini et al. is thus concerned with an application in nuclear medicine where the intrinsic radioactivity of a substance is put to use. The substance in Serafini et al.'s case, Iodine-123-labeled-Rose Bengal, *emits* radiation which Serafini et al. reports on for imaging purposes. As seems appropriate, this article appears in a journal directed to nuclear medicine. Fundamentally, this reference indicates the advantage of one radioactive form of Rose Bengal over another, in forming images from the radioactive emissions. There is no disclosure or suggestion of a radiosensitizer agent, ionizing radiation, or treatment of cancers or tumors.

3. The Examiner's Rejection

The Examiner admits, as she must, that this reference fails to disclose the discovery of halogenated xanthenes as a radiosensitizer agent. Specifically, in the Office Action of September 13, 2001, the Examiner concurs with Appellants that Serafini et al. does not disclose the use of Rose Bengal as a radiosensitizer agent for use with ionizing radiation to treat cancer or tumors. Exhibit E, p. 6. The Examiner further concurs that Serafini et al.'s use is completely different from the use as a radiosensitizer agent of Claim 1. Id. The Examiner, however, argues that Serafini et al. discloses the same Rose Bengal having the same structure and functional properties as the radiosensitizer agent of Claim 1. Id. The Examiner apparently believes that is enough to reject the claims. The Examiner also relies upon the "reasons of record." Id. at p. 3.

4. Serafini et al. Does Not Disclose or Suggest All The Elements Of The Rejected Claims

The rejection of Claims 1-3 and 5-8 as being anticipated by Serafini et al. is plainly erroneous, as Serafini et al. fails to disclose all of the elements of the claims rejected thereover.

Independent Claim 1 specifically recites:

> "A *radiosensitizer agent* for *treatment* of cancer and tumors, said radiosensitizer agent comprising a halogenated xanthene, said halogenated xanthene *interacting with ionizing radiation applied to* the cancer or tumor to enhance the therapeutic efficacy of said ionizing radiation." (emphasis added)

The claim is not merely directed to a halogenated xanthene or Rose Bengal, as the Examiner argues. As explained supra, it is directed to a special class of material, a radiosensitizer agent, which comprises a halogenated xanthene and which interacts with ionizing radiation to treat cancer and tumors.

Claim 1 specifically recites "A radiosensitizer agent" in the preamble. The Court of Appeals for the Federal Circuit has clearly stated that the preamble may be a limitation of the claim when it is required to confer meaning on the claim. Phillips Petroleum Company v. Huntsman Polymers Corp., 48 USPQ2d 1161 (Fed. Cir. 1998). See also Union Oil, 208F.3d at 995-996, 54 USPQ 2d at 1231-1232. This is true even when the preamble is stated in the form of "A ____ comprising". Id.

This case is similar to that in the Union Oil case, 208 F.3d 989. In that case, the preamble of the claim stated "An unleaded gasoline fuel suitable for combustion in an automotive engine." The district court concluded that the claims cover fuels that will regularly be used in autos, not all that conceivably could be used, and therefore found that the claims cover a narrow class of fuel compositions, namely only standard automotive gasoline. The district court excluded from the claim scope a broader class of petroleum formulations such as aviation fuels or racing fuels. As a result,

the court found that references from these other fields did not invalidate the claims. The Federal Circuit agreed with this analysis.

Hence, in contrast to independent Claim 1, Serafini et al. does not disclose or suggest:

- a radiosensitizer agent,
- a radiosensitizer agent for treatment of cancer and tumors,
- a radiosensitizer agent comprising a halogenated xanthene, or
- a halogenated xanthene that interacts with ionizing radiation applied to the cancer or tumor to enhance the therapeutic efficacy of the ionizing radiation.

Merely because Serafini et al. discloses Rose Bengal and one use of Rose Bengal does not mean that it discloses any of the above items. As explained above, the FDA requires labeling for drugs, such as radiosensitizer agents, including information on intended uses. Hence, when any drug is sold as a radiosensitizer or radiosensitizer agent for interaction with ionizing radiation for treatment of cancers or tumors, its labeling must state that it is such a radiosensitizer. This would apply to Rose Bengal or another halogenated xanthene sold as a radiosensitizer for interaction with radiation. As explained above, any Rose Bengal sold for use for the diagnostic purposes described in Serafini et al., and not for therapeutic interaction with ionizing radiation, would have a different labeling and thus be a different drug and distinctly different commercial entity.

Hence, Serafini et al. clearly does not disclose or suggest all of the elements of independent Claim 1, as will be shown in even more detail below.

In the Office Action of September 13, 2001, the Examiner also states that the rejected claims are anticipated by Serafini et al. for the reasons of record which include the statement that Serafini et al. teaches Rose Bengal:

> "which has an inherent capacity to rapidly and efficiently concentrate into cellular molecules upon exposure to ionizing radiation; thus, a radiosensitizer or a pharmaceutical agent which in combination with ionizing radiation, finds use in treating diseased tissue with overall reduction in radiation exposure to treat diseases such as cancer or tumor. Specifically, Applicant claims a radiosensitizer agent, halogenated xanthene, a known product clearly taught by Serafini et al. and which has the inherent property to concentrate into diseased tissue (as shown by Serafini et al.). Therefore, the product of the present invention is concluded to be inherently anticipated by Serafini et al." (See Exhibit B)

Appellants strongly disagree with the Examiner's conclusion and note that the Examiner has made no specific citation to any disclosure in Serafini et al. where "concentrate[ing] into cellular molecules upon exposure to ionizing radiation" is shown. Nor has the Examiner shown a disclosure in this reference of any halogenated xanthene which "treat diseases" or of a radiosensitizer agent for treatment of cancer and tumors. Indeed, the Examiner has not pointed out where halogenated xanthenes are identified as "a radiosensitizer agent." Basically, the Examiner's position is that Serafini et al. discloses Rose Bengal which is enough. But that is not nearly enough to disclose all of the elements of the invention claimed in independent Claim 1.

More specifically, Serafini et al., does not disclose or suggest a radiosensitizer agent since no exogenous radiation is applied therapeutically, diagnostically or otherwise, to the tissue containing the Rose Bengal. The fact that Serafini et al. teaches that Rose Bengal could be tagged with radioactive Iodine-123 (page 630, col. 1) does not mean that Rose Bengal is a radiosensitizer. And the teaching that Iodine-123 can be a useful substitute for ^{131}I as a label for radiopharmaceuticals likewise is not a disclosure that Rose Bengal is a radiosensitizer. Additionally, Appellants find no suggestion of any therapeutic modality in Serafini et al. Further, any radiation emanating from I-123

or I-131 that hits the tissue as a result of the radioactive decay of the imaging agent would not be regarded as therapeutically applied "ionizing radiation" by those skilled in the art.

Instead, the reference actually teaches away from the claimed invention. A central theme of Serafini et al. is the use I-123 instead of I-131 so as to *minimize the effects of spontaneously emitted radiation* to tissues containing diagnostic quantities of radiolabelled Rose Bengal (as well as to minimize or avoid damage to surrounding tissues and to tissues containing degradation products of such radiolabelled Rose Bengal). For example, in the abstract, Serafini et al. (Exhibit K) states:

> "The overall reduction in imaging time and radiation exposure...should greatly improve our diagnostic capabilities in evaluating the jaundiced patient."

In the second paragraph (p. 629), Serafini et al. states:

> "Because of the relatively high absorbed radiation dose produced by the beta decay and the 8-day half life of [radiolabelled] ^{131}I rose bengal, we are limited in the amount of activity [of radiolabelled rose bengal] that can be administered to the patient..."

Finally, in the discussion (p. 631-632), Serafini et al. states:

> "Iodine-123 when compared with ^{131}I has several desirable physical properties.... The relatively short 13- hr half-life of ^{123}I decreases patient exposure [to radiation]..."

Thus, one of the fundamental concepts Serafini et al. is directed to is minimizing the effects of radiation in tissues containing Rose Bengal. This is completely the opposite of what is claimed and taught in the present application, i.e. a radiosensitizer agent which seeks to *maximize the effects of ionizing radiation* in tissues containing Rose Bengal or another hydrogenated xanthene.

Further, the Examiner has absolutely no basis for suggesting that Serafini et al. teaches that Rose Bengal targets diseased tissue (such as that of cancerous tumors). Serafini et al. instead illustrates that retention of Rose Bengal in portions of the liver and gall bladder can be used to

diagnose the function of these particular organs. Specifically, anomalous patterns of retention of Rose Bengal, as illustrated by Figs. 1 and 2 of Serafini et al., can be used to identify and diagnose areas of abnormal excretion. In normal organs, Rose Bengal is expected to exhibit a characteristic concentration half-life and should exhibit substantially uniform distribution (i.e., isotropic distribution) throughout the organ. Upon intravenous administration, a bolus of Rose Bengal perfuses a given organ, then gradually washes out as the bolus is cleared from the blood stream (or in the case of the liver or gall bladder, the Rose Bengal is excreted homogeneously through normal physiologic functions of the organ, for example into the bile). In a diseased liver or gall bladder, anisotropic retention in certain portions of the organ is characteristic of reduced excretory function of such portions. Accordingly, Rose Bengal, as used by Serafini et al., is not targeting these diseased portions, but instead is merely backing up in these portions (i.e., it is not being excreted through such portions at a normal rate). It is further noted that because it is based on backing up in certain blood purifying organs, instead of targeting cancer or tumors, only the liver and gall bladder are discussed in Serafini et al.

In short, this reference uses the radioactive quality of Iodine -123-Rose Bengal for diagnostic imaging of the liver and gall bladder -- just as its title states.

Therefore, as explained above and contrary to the Examiner's argument, the invention claimed in independent Claim 1 is not merely Rose Bengal. Instead, the claimed invention is directed to a radiosensitizer agent for treatment of cancer and tumors comprising a halogenated xanthene which interacts with ionizing radiation to enhance the therapeutic efficacy of the ionizing radiation to treat cancer and tumors. Appellants are unaware of any such prior disclosure.

Accordingly, for at least the above-stated reasons, there is no disclosure or suggestion in Serafini et al. of (1) Rose Bengal or any other halogenated xanthene[4] as a radiosensitizer agent, (2) a radiosensitizer agent for any therapeutic treatment of cancers or tumors, (3) a radiosensitizer agent comprising a halogenated xanthene; or (4) a halogenated xanthene in a radiosensitizer agent, that interacts with ionizing radiation applied to the cancer or tumor to enhance the therapeutic efficacy of the ionizing radiation. Serafini et al.'s mere disclosure of Rose Bengal is insufficient to disclose or suggest the elements of independent Claim 1 and in fact, appears to have almost no relevance to the present invention and the recited claims.

For the above-stated reasons, the claims of the present application are neither anticipated nor obvious over Serafini et al., and the rejection thereover should be reversed.

C. CLAIMS 1-3 AND 5-9 ARE NOT ANTICIPATED BY NECKERS

In the Office Action of September 13, 2001, the Examiner rejected Claims 1-3, 5-9 and 12-13 [5] under 35 USC §102 as being "inherently anticipated by Neckers." This rejection is also erroneous, as Neckers is clearly different than the claimed invention and fails to disclose or suggest all of the elements of the rejected claims.

[4] Serafini et al. mentions only Rose Bengal and does not disclose or suggest any other halogenated xanthene, nor any functional derivative thereof.

[5] Appellants canceled Claims 12 and 13 in Amendment B, and therefore the rejection of claims 12 and 13 is moot.

1. Neckers

Neckers (Exhibit L), according to its own summary, reports and compares the optical properties, photochemical reactivity, and photophysical parameters of known derivatives of Rose Bengal. As such, it describes many fundamental chemical and physical properties of a number of the halogenated xanthenes, particularly Rose Bengal. Neckers mentions use "as a diagnostic probe," apparently in reference to Eosin. Neckers at page 3. Among other things, Neckers further mentions a "fluorescence spectrum" of a certain salt, noting that it has "a broad triad of peaks centered at 583nm (Table 3)." Id. at page 13. Neckers also mentions optical absorption and emission spectra, intermolecular energy transfer as measured by singlet oxygen yields, and intramolecular self-quenching. Id. at pages 18-21. A further discussion of quenching appears at pages 22-24 of Neckers.

2. The Examiner's Rejection and Comments

In the Office Action, the Examiner makes comments in support of this rejection similar to the ones given in support of her rejection over Serfani et al. More specifically, the Examiner concurs that Neckers does not disclose use of Rose Bengal as a radiosensitizer agent for use with ionizing radiation to treat cancer or tumors. Exhibit E, p. 7-8. The Examiner also concurs that the Neckers use is completely different from use as the radiosensitizer agent of Claim 1. Id.

Nevertheless, the Examiner rejects independent Claim 1, and dependent Claims 2, 3 and 5-9, as anticipated by Neckers and argues that Neckers teaches the same Rose Bengal having the same structure and functional properties as the radiosensitizer agent recited in Claim 1 which she apparently believes is enough to anticipate the Claims. Id. The Examiner also rejects the claims for the "reason of record." Id. at p. 3.

3. Neckers Fails to Disclose or Suggest the Claimed Invention

As explained above for the rejection based on Serafini et al., independent Claim 1 of the present application is not merely directed to a halogenated xanthene or Rose Bengal, as the Examiner argues. Instead, it is directed to a radiosensitizer agent comprising a halogenated xanthene which interacts with ionizing radiation applied to a cancer or tumor to enhance the therapeutic efficacy of the ionizing radiation. Neckers does not disclose or suggest a radiosensitizer agent; a radiosensitizer agent comprising a halogenated xanthene, or a halogenated xanthene that interacts with ionizing radiation to enhance the therapeutic efficacy of the ionizing radiation. Hence, Neckers does not disclose or suggest the invention or every element of independent Claim 1, and for similar reasons as discussed above for Serafini et al. the radiosensitizer agent of independent Claim1 and those claims dependent thereon is patentable distinguishable over Neckers.

For example, in accordance with FDA regulations, the radiosensitizer agent of Claim 1, which interacts with ionizing radiation, would be in an entirely different classification than the laboratory reagent which interacts with visible light, disclosed in Neckers. Therefore, in accordance with Union Oil *supra*, radiosensitizers are a narrow class, and laboratory reagents such as in Neckers should be excluded from this class and not permitted to invalidate the radiosensitizers of Claim 1. Hence, the basis of this rejection, as stated in September 13, 2001 Office Action, is erroneous, and the rejection should be reversed.

Furthermore, one of ordinary skill in the art would not be led to such radiosensitizer agents based on references, such as Neckers, pertaining to the photochemical/laboratory reagent use of Rose Bengal with visible light activation. The fundamental physics of interaction with visible light and with ionizing radiation are completely different. More specifically, visible light interaction of the

halogenated xanthenes results in photocatalytic conversion of triplet oxygen into cytotoxic singlet oxygen, while interaction of any radiodense material, such as the halogen atoms contained in the halogenated xanthenes, with ionizing radiation results in cell necrosis from absorbance of and spontaneous re-emission of such ionizing radiation from such radiodense materials at lower, more cytotoxic energies. Further, as key features characterizing the interaction of visible light with the halogenated xanthenes vary with the overall molecular structure of such compounds (as evidenced by variations in optical spectroscopic properties, such as absorbance maxima and excited state lifetime, with changes in location of halogen substitution, as clearly taught throughout Neckers), while the key features characterizing the interaction of ionizing radiation with such compounds is affected only by absolute halogen content (and not by their location within the molecule), the compound disclosed in Neckers and the radiosensitizer agent of Claim 1 are fundamentally different (e.g., with visible light, the halogens serve to modify the photochemical and photophysical properties of the molecule, while upon interaction with ionizing radiation, the halogenated xanthene molecule serves as a vehicle for concentration and delivery of the active halogens).

The Examiner's reasons of record are also erroneous. They include the statement that Neckers teaches Rose Bengal:

> "which has an inherent capacity to rapidly and efficiently concentrate into cellular molecules upon exposure to ionizing radiation; thus, a radiosensitizer or a pharmaceutical agent which in combination with ionizing radiation, finds use in treating diseased tissue. Specifically, Applicant claims a radiosensitizer agent, halogenated xanthene, a known product clearly taught by Neckers and which has the inherent property to concentrate into diseased tissue. Therefore, the product of the present invention is concluded to be inherently anticipated by Neckers."

Final Rejection, Exhibit B, pp. 9-10. The Examiner's conclusion, along with many of the Examiner's assertions concerning the facts about this reference, are clearly incorrect.

The Examiner's reliance on this reason of record that Neckers inherently discloses Rose Bengal as a radiosensitizer for use with ionizing radiation is clearly contrary to her admission in the September 13, 2001 Office Action (Exhibit E) that Neckers does not disclose Rose Bengal as a radiosensitizer agent. In fact, it cannot be disputed that Neckers fails to provide any teaching or suggestion of a radiosensitizer agent or a radiosensitizer agent comprising a halogenated xanthene.

In addition, Appellants find no disclosure or suggestion in Neckers of Rose Bengal or another halogenated xanthene interacting with ionizing radiation.

Further, Appellants find no disclosure in Neckers of radiosensitizer agents for treatment of cancer or tumors or such agents interacting with ionizing radiation applied to the cancer or tumor to enhance the therapeutic efficacy of the ionizing radiation, as required in independent Claim 1of the present application. Neckers describes only Rose Bengal and *optical* radiation (i.e., ultraviolet, visible and near infrared light), not *ionizing* radiation.[6]

The Examiner also made reference to Rose Bengal "concentrating into cellular molecules upon exposure to ionizing radiation". Appellants do not find that disclosure in the reference, and the Examiner has not identified its location so Appellants can consider it more fully.

[6] Appellants note that Neckers is published in *J. Photochem. Photobiol.*, a journal that has a topical focus on the interaction of optical radiation (i.e., ultraviolet, visible and near infrared light) with matter, rather than on the interaction of x-rays, gamma-rays, and other high energy ionizing radiation with such matter. Such a journal is appropriate for the subject matter of Neckers, and is further indicative that Neckers concerns the interaction of *optical* radiation, not *ionizing* radiation or radiosensitizer agents.

In short, Appellants see nothing whatsoever in Neckers to indicate or suggest a radiosensitizer agent or a radiosensitizer agent comprising a halogenated xanthene and ionizing radiation applied to cancer or tumors to enhance the therapeutic efficacy of the ionizing radiation, as specifically recited in Claim 1. Instead, Neckers appears to be cited merely because it discloses Rose Bengal, and for no other reason. Merely identifying Rose Bengal or other halogenated xanthenes as known compounds is far short of meeting all of the limitations of the rejected claims.

Accordingly, for at least the above-stated reasons, Neckers does not disclose or suggest the radiosensitizer agent of the claims of the present application, and the claims are clearly not anticipated by Neckers but are patentable thereover. Therefore, the rejection of these claims over Neckers should be reversed.

D. THE §103 REJECTIONS SHOULD BE REVERSED

The Examiner also had two rejections of the claims under 35 USC §103. As shown below, in each of these rejections, the Examiner has failed to make a prima facie case of obviousness and has used hindsight reconstruction to improperly combine references to arrive at the claimed inventions. As a result, the rejections are erroneous and should be reversed.

1. Under The Law, The PTO Is Required To Establish A Prima Facie Case Of Obviousness

Under 35 U.S.C. §103, the burden is on the PTO to produce evidence that the claimed invention is prima facie obvious. In re Rijckaert, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993); In re Fine, 837 F.2d 1071, 1074, 5 USPQ2d 1596, 1598 (Fed. Cir. 1988). If the PTO fails to make out a prima facie case of obviousness, then the rejection is improper, should be overturned,

and Applicant is entitled to a patent. Rijckaert, 9 F.3d at 1532, 28 USPQ2d at 1956; In re Nielson, 816 F.2d 1567, 1572, 2 USPQ2d 1525, 1528 (Fed. Cir. 1984); In re Gordon, 733 F.2d 900, 902, 221 USPQ 1125, 1127 (Fed. Cir. 1984).

2. The Examiner's Rejection of Claims 4, 14 and 18-10 is in Error

Five claims are at issue for this first §103 rejection. Specifically, the Examiner rejects Claims 4, 14 and 18-20 under 35 USC §103 as being unpatentable over Serafini et al. or Neckers in view of Khaw et al. for reasons of record. This rejection is erroneous as the Examiner has failed to make out a prima facie case of obviousness. Further, the references fail to disclose or suggest the claimed invention, and the combination of references is improper. As a result, the rejection should be reversed.

As explained above, neither of the primary references, Serafini et al. and Neckers, discloses nor suggests any radiosensitizer or radiosensitizer agent, and certainly do not disclose nor suggest the radiosensitizer as claimed in the present application. As a result, even if these references were combined with Khaw et al.[7], the combination still would fail to disclose or suggest a radiosensitizer agent comprising a halogenated xanthene, or a radiosensitizer agent comprising a halogenated xanthene that interacts with ionizing radiation applied to the cancer or tumor to enhance the therapeutic efficacy of the ionizing radiation, as claimed in the present application. As discussed above, neither of these primary references discloses or suggests that Rose Bengal or halogenated

[7]As discussed *infra*, such a combination to arrive at the claimed present invention is improper.

xanthenes are effective as a radiosensitizer agent for interaction with ionizing radiation. Hence, the Examiner has failed to make a prima facie case of obviousness.

The Examiner, however, makes the same argument as above, re that Serafini et al. and Neckers, combined with Khaw et al. (Exhibit K) disclose the same Rose Bengal as recited in the claims. For the reasons explained above, neither Serafini et al. nor Neckers discloses or suggests the claimed invention. Khaw et al. does not disclose a halogenated xanthene. Adding it to either reference in combination does not cure these shortcomings, for Khaw et al., as will be explained below, also fails to disclose the omitted features of independent Claim 1. Since the features of independent Claim 1 are patentably distinct over the hypothetical combination, all the claims dependent thereon are patentably distinct also.

The dependent claims add further distinguishing features. For example, rejected dependent Claims 4 and 14 concern targeting while rejected Claims 18-20 concern other dependent features, encapsulation being one. This allegedly is the reason for the citation of Khaw et al.[8] Khaw et al. fails to disclose any of the claimed features of the present application.

Instead, Khaw et al. discloses methods of manufacture and use of immunoliposomes (i.e., liposomes doped on their outside surface with an immunoactive moiety, such as an antibody). Such immunoliposomes are purported to provide means for targeted delivery of their contents to various immunologic targets (such as certain cancer cells) (see, e.g. col. 6, lns. 44-50 of Khaw et al.). Use of such immunoliposomal delivery means is taught for use with therapeutic radioactive iodine (see col. 11, lns. 37-46) and for various "radiopaque" materials (see col. 11, lns 53-64). Specific

[8]One has to go back to the first office action of August 29, 2000 (Exhibit A) to find the stated reasoning.

examples of such use with contrast agents is given in Table I of Khaw et al. Additional uses with radiosensitizing compounds is disclosed at col. 13, lns. 62-65. This is further discussed at col. 16, lns. 9-34 and lns. 52-60, and at col. 17, lns. 13-16, all in Khaw et al.

Notably, Khaw et al. teaches that such delivery is based primarily on action of a specific affinity reagent (i.e., the immunoactive moiety) with specific intracellular antigens (as taught at col. 3, lns. 14-21). This fundamental concept is markedly different than the present invention, which is directed to new radiosensitizer agents comprising halogenated xanthenes that exhibit intrinsic targeting for certain types of cells and tissues along with certain structural components of such cells and tissues (see, e.g. p. 6, ln. 12 - p. 7, ln. 23, of the present application). Therefore, complex adjuvants are not required for transport and delivery, but instead simple adjuvants (*e.g.*, simple liposomal formulations such as those claimed in the present application) can be used in the formulation and optimization of such agents for certain physical targeting purposes (*see e.g.* p. 7, lns. 17-21 of the present application). As a result, Khaw et al. teaches away from the subject matter of the present invention by requiring use of specific immunologic delivery means and liposomal packaging for successful transport and delivery of radiocontrast or radiosensitizer agents to diseased tissue.

Further, Khaw et al. does not disclose nor suggest radiosensitizer agents comprising halogenated xanthenes.

Where none of the references in the hypothetical combination teach radiosensitizer agents comprising halogenated xanthenes that interact with ionizing radiation, combining them fails to produce the missing teachings. Even when combined, these three references fail to disclose or

suggest, either explicitly or otherwise, the radiosensitizer agent comprising a halogenated xanthene for interaction with ionizing radiation of the claims of the present application.

Further, any combination of these references in an effort to reach the rejected claims is improper hindsight reconstruction. Appellants do not admit that such a combination is proper. In fact, it is respectfully submitted that the only way that these references could be combined to arrive at the claimed invention is through the use of hindsight reconstruction using the claimed invention as a blueprint. Neither Serafini et al. nor Neckers teaches or suggests a radiosensitizer agent comprising a halogenated xanthene. Khaw et al. provides no teaching with regard as to discovering new radiosensitizers. Hence, one skilled in the art would not be led by Khaw et al. to conclude that the disclosures of Serafini et al. or Neckers have any relevance to radiosensitizers (which they do not). Further, each reference is in a completely unrelated field. Hence, it is difficult to understand how such references could be combined to arrive at the claimed invention when each is in a different field and none disclose or suggest the important features of the claimed invention. As a result, the Court of Appeals for the Federal Circuit has held that such a combination is improper and cannot form the basis of a §103 obviousness rejection. *See* Ecolochem, Inc. v. Southern California Edison Company, 56 USPQ2d 1065, 1072-1076 (Fed. Cir. 2000). Hence, the rejection based thereon should be reversed.

Therefore, the claims are patentable over these references, and the rejection of these claims should be reversed.

3. The §103 Rejection of Claims 10, 51, 52 and 56-57 Is Also In Error

The Examiner also gave a second §103 rejection directed to Claims 10, 51, 52 and 56-57 under 35 USC §103 as being unpatentable over Serafini et al. or Neckers in view of Norman (1991) for reasons of record. This rejection also is erroneous and should be reversed.

As the Examiner recognizes, neither Serafini et al. nor Neckers discloses or suggests a radiosensitizer agent comprising a halogenated xanthene for treatment of cancer and tumors using ionizing radiation, wherein halogenated xanthene is activated using x-rays having an energy greater than 30 keV (Claim 10) or wherein ionizing radiation is approximately greater than or equal to 1 keV and less than or equal to approximately 1000 MeV (Claims 51 and 52) or wherein ionizing radiation is x-rays (Claim 56) and has an energy of between 30 kiloelectron volts and 1000 megaelectron volts (Claim 57).

As explained above, neither of these references have any disclosure or suggestions at all of radiosensitizer agents or ionizing radiation. Hence, the Examiner has failed to make a prima facie case of obviousness. The Examiner, however, uses the same argument as above, i.e., that Serafini et al. and Neckers merely disclose Rose Bengal and that is enough. As explained in depth above, the claims require much more than Rose Bengal, and the additional recitations in the claims are not disclosed or suggested by either of these references.

The Examiner cites Norman (Exhibit N) in the Final Rejection (Exhibit B) (and maintains that rejection in the September 13, 2001 Office Action) as teaching a radiosensitizer agent and that it would be obvious to substitute the Rose Bengal of Serafini et al. or Neckers for the iodinated contrast agent taught by Norman.

However, these references, even if combined[9], yield a combination that would still fail to disclose or suggest the claimed invention, i.e. a radiosensitizer agent comprising a halogenated xanthene which is activated using x-rays having an energy greater than 30 keV (independent Claim 10) or a radiosensitizer comprising a halogenated xanthene and ionizing radiation of approximately greater than or equal to 1 keV and less than or equal to 100 meV (independent Claim 51). As explained above, neither Serafini et al. nor Neckers discloses or suggests these features.

Norman also fails to disclose these features of the present invention. In fact, Norman actually teaches away from the claimed radiosensitizer agent of the present application in the teaching in the reference of three possible ways to increase the efficacy of radiosensitization: (1) irradiation at specific energies that are maximally absorbed by the radiosensitizer; (2) use of a different compound with higher radiodensity (i.e., gadolinium); or (3) use of complex radiosensitizer conjugates that are directly incorporated into tumor cells (see "Discussion" on page S120 of Norman). In each of these elements, Norman teaches away from the subject matter of the claims of the present application.

In the first example in Norman, the reference states that practitioners can use a specific activation energy of 32 keV but that such energy will have a poor penetration through the skull and brain to a tumor. Hence, Norman admits that this technique is undesirable. Further, there is no teaching in this example of a halogenated xanthene nor of a halogenated xanthene in a radiosensitizer agent for activation by x-rays or for treatment using ionizing radiation, as in the claims of the present invention. In contrast to Norman, the present application teaches that the halogenated xanthenes are compatible with a broad range of activation energies (see, for example, p. 9, line 20 - p. 10,

[9] As explained *infra*, such a combination is improper.

line18 which discuss the suitability of halogenated xanthenes in radiosensitizers agents and that they do not suffer the infirmaries of the Norman technique).

In the second example in Norman, the reference requires that practitioners use an exotic radiodense material, gadolinium. In contrast, the claims of the present application are directed to a radiosensitizer agent comprising a halogenated xanthene which overcomes the shortcomings of earlier classes of radiosensitizers. Halogens (i.e., fluorine, chlorine, bromine, and iodine) and the claimed halogenated xanthenes as taught in the present application, are not exotic radiodense materials. Furthermore, Gadolinium is not a halogenated xanthene, and no known halogenated xanthene contains gadolinium.

In the third example in Norman, the reference teaches the use of complex radiosensitizer/nucleic acid conjugates for enhanced agent incorporation into target cells. However, Norman specifically states that such means are "a difficult and expensive method as compared to injecting [more radiosensitizer]." (p. S121) Thus, Norman teaches away from one of the benefits of the subject matter of the present application by recommending that practitioners *use more agent*, rather than *a better agent* that exhibit good intrinsic targeting. Further, Norman notes that a clear obstacle to proceeding with his method is toxicity to the patient! Hence, Norman appears to be admitting that this teaching is infirm.

In contrast, the halogenated xanthene in the radiosensitizer agents of the rejected claims of the present application exhibit intrinsic targeting and incorporation into tumor and other diseased tissue (see, for example, p. 6, line 13 - p. 7, line 23). Such intrinsic targeting does not require additional difficult or expensive methods for manufacture or use. Hence, these agents are far superior to those contemplated by Norman. Further, since the halogenated xanthenes have a

significant regulatory history and low agent cost (see p. 8, lines 2-4, of the present application), such compounds do not have an expensive cost associated with their use in radiosensitization, as reported by Norman. Furthermore, numerous toxicology studies have shown that this class of compounds (i.e., halogenated xanthenes) is non-toxic, except when provided in the high concentration in the cancer or tumors in the present invention.

Hence, even if these three references were combined, the combination still fails to disclose or suggest the claimed invention.

Further, the combination of these references is improper. More specifically, based on these substantive contradictions between the teachings of Norman and the claims of the present application, Norman would fail to motivate one of ordinary skill in the art to arrive at a radiosensitizer agent comprising a halogenated xanthene, as specifically claimed in the present application. Hence, one skilled in the art would not combine Norman with either Serafini et al. or Neckers, and none of the references suggest such a combination, as required by the Court of Appeals for the Federal Circuit. Ecolochem, 56 USPQ2d at 1072-1076. This is particularly true where the art teaches away from the claimed invention. Id. at page 1075.

Again, it is difficult to understand how a combination of references that nowhere disclose or suggest important features of the invention could suggest or lead the ordinary skilled artisan to the invention itself. Norman says nothing about halogenated xanthenes. Serafini et al. is concerned with scintillation photography using a radioactive form of Rose Bengal, but says nothing about using halogenated xanthenes in a radiosensitizer agent or ionizing radiation. Neckers discusses optical properties of Rose Bengal but again fails to suggest anything regarding a radiosensitizer agent or ionizing radiation. Since Serafini et al., Neckers, and Norman all fail to suggest halogenated

xanthenes in a radiosensitizer agent for treating cancer and tumors and that the halogenated xanthene is activated using x-rays as in Claim 10 or uses ionizing radiation for treatment of cancers or tumors as in Claim 51, Appellants find no teaching from which these references can be combined to arrive at the present invention. Certainly, Appellants see no prima facie showing by the Examiner for which Appellants have to rebut. Further, Appellants submit that, even if these references could be combined arguendo, when combined none of them discloses or suggests these features of the claimed invention. Hence, even when combined, they fail to disclose the invention of the rejected claims, and the invention would not have been obvious in view of such a combination.

Moreover, since the 1991 publication date indicated for Norman, seven years passed before the filing of Appellants' parent application in 1998, with no publication nor public use (so far as Appellants are aware) of using Rose Bengal or any other halogenated xanthene in a radiosensitizer agent for treating tumors or cancer tissue with ionizing radiation or activation by x-rays. Given that cancer research is of global importance and focus, the passage of these several years following the publication of Norman, with no one appreciating the invention now claimed, is strong evidence of the non-obviousness of the claimed subject matter.

Accordingly, this rejection should be reversed.

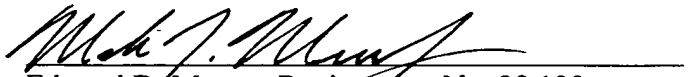
E. CONCLUSION

For the reasons stated above, we earnestly and respectfully submit that the rejections under 35 USC §102 and §103 of the claims of the present application as being anticipated by and unpatentable over the references cited by the Examiner in the Office Action are erroneous. The references cited by the Examiner do not withstand close examination: they do not disclose nor

suggest many elements recited in the (separately-argued) claims. Further, the Examiner fails to address where many of the elements of the dependent claims are shown in any of the cited references. Hence, the rejection of these claims should be withdrawn or reversed, and the claims allowed.

Accordingly, Appellants requests that this Appeal be sustained in all respects, and that all rejections and objections in the Office Action be reversed.

Respectfully submitted,



Edward D. Manzo, Registration No. 28,139
Mark J. Murphy, Registration No. 34,225

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May 13, 2002

IX. APPENDIX

APPEALED CLAIMS (37 CFR 1.192 (C)(7))

The text of the claims on appeal is as follows:

1. A radiosensitizer agent for treatment of cancer and tumors, said radiosensitizer agent comprising a halogenated xanthene, said halogenated xanthene interacting with ionizing radiation applied to said cancer or tumor to enhance the therapeutic efficacy of said ionizing radiation.

2. The radiosensitizer agent of Claim 1, wherein said halogenated xanthene comprises Rose Bengal.

3. The radiosensitizer agent of Claim 1, wherein said halogenated xanthene comprises 4,5,6,7-Tetrabromoerythrosin.

4. The radiosensitizer agent of Claim 1 wherein said halogenated xanthene includes as a functional derivative at least one targeting moiety selected from the group consisting of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), amino acids, proteins, antibodies, ligands, haptens, carbohydrate receptors or complexing agents, lipid receptors or complexing agents, protein receptors or complexing agents, chelators, encapsulating vehicles short- or long-chain aliphatic or aromatic hydrocarbons, aldehydes, ketones, alcohols, esters, amides, amines, nitriles, and azides.

5. The radiosensitizer agent of Claim 1 wherein said halogenated xanthene also is an imaging contrast agent.

6. The radiosensitizer agent of Claim 5 wherein said halogenated xanthene acts as an imaging contrast agent for computed axial tomography.

7. The radiosensitizer agent of Claim 5 wherein said halogenated xanthene acts as an imaging contrast agent for X-ray imaging.

8. The radiosensitizer agent of Claim 1 wherein said halogenated xanthene includes at least one halogen selected from the group consisting of iodine and bromine.

9. The radiosensitizer agent of Claim 1 wherein said halogenated xanthene is selected from the group consisting of Phloxine B, Erythrosin B and Eosin Y.

10. A radiosensitizer agent for treatment of cancer and tumors using ionizing radiation, said radiosensitizer agent comprising a halogenated xanthene wherein said halogenated xanthene is activated using x-rays having an energy greater than 30 keV.

14. The radiosensitizer agent of Claim 1 wherein at least one biological targeting moiety is attached to said halogenated xanthene to enhance targeting of said halogenated xanthene to biologically sensitive structures of said cancer or tumors.

15. The radiosensitizer agent of Claim 1 wherein at least one chemical targeting moiety is attached to said halogenated xanthene to enhance targeting of said halogenated xanthene to biologically sensitive structures of said cancer or tumors.

18. The radiosensitizer agent of Claim 1 wherein said halogenated xanthene is encapsulated in a delivery vehicle.

19. The radiosensitizer agent of Claim 18 wherein said halogenated xanthene is Rose Bengal.

20. The radiosensitizer agent of Claim 18 wherein said delivery vehicle is selected from the group consisting of micelles, nanoparticles and liposomes.

51. A radiosensitizer agent for treatment of cancer and tumors using radiosensitization or ionizing radiation, said radiosensitizer agent comprising a halogenated xanthene wherein said ionizing radiation is approximately greater than or equal to 1 keV and less than or equal to approximately 1000 MeV.

52. The radiosensitizer agent of Claim 1 wherein said ionizing radiation is approximately greater than or equal to 1 keV and less than or equal to approximately 1000 MeV.

55. The radiosensitizer agent of Claim 1 wherein said halogenated xanthene includes as a functional derivative at least one targeting moiety selected from the group consisting of hydrophilic and hydrophobic moieties.

56. The radiosensitizer agent of Claim 1 wherein said ionizing radiation comprises x-rays.

57. The radiosensitizer agent of Claim 56 wherein said x-rays have an energy between 30 kiloelectron volts and 1000 megaelectron volts.

60. The radiosensitizer agent of Claim 10, wherein said halogenated xanthene comprises Rose Bengal.

61. The radiosensitizer agent of Claim 10 wherein said halogenated xanthene includes as a functional derivative at least one targeting moiety selected from the group consisting of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), amino acids, proteins, antibodies, ligands, haptens, carbohydrate receptors or complexing agents, lipid receptors or complexing agents, protein receptors or complexing agents, chelators, encapsulating vehicles short- or long-chain aliphatic or aromatic hydrocarbons, aldehydes, ketones, alcohols, esters, amides, amines, nitriles, and azides.

62. The radiosensitizer agent of Claim 10 wherein said halogenated xanthene also is an imaging contrast agent.

63. The radiosensitizer agent of Claim 10 wherein said halogenated xanthene is encapsulated in a delivery vehicle.

64. The radiosensitizer agent of Claim 51, wherein said halogenated xanthene comprises Rose Bengal.

65. The radiosensitizer agent of Claim 51 wherein said halogenated xanthene includes as a functional derivative at least one targeting moiety selected from the group consisting of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), amino acids, proteins, antibodies, ligands, haptens, carbohydrate receptors or complexing agents, lipid receptors or complexing agents, protein receptors or complexing agents, chelators, encapsulating vehicles short- or long-chain aliphatic or aromatic hydrocarbons, aldehydes, ketones, alcohols, esters, amides, amines, nitriles, and azides.

66. The radiosensitizer agent of Claim 51 wherein said halogenated xanthene also is an imaging contrast agent.

67. The radiosensitizer agent of Claim 51 wherein said halogenated xanthene is encapsulated in a delivery vehicle.

suggest many elements recited in the (separately-argued) claims. Further, the Examiner fails to address where many of the elements of the dependent claims are shown in any of the cited references. Hence, the rejection of these claims should be withdrawn or reversed, and the claims allowed.

Accordingly, Appellants requests that this Appeal be sustained in all respects, and that all rejections and objections in the Office Action be reversed.

Respectfully submitted,



Edward D. Manzo, Registration No. 28,139
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May 13, 2002

Exhibit A

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
09/382,622	08/25/99	DEES H	PHO-107-DIV

HM12/0829

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EXAMINER	
GABEL, G	
ART UNIT	**PAPER NUMBER**
1641	

DATE MAILED: 08/29/00

RECEIVED
AUG 31 2000
COOK, ALEX, McFARRON,
MANZO, CUMMINGS & MEHLER

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Photocopy

CASE PHO-107 DIV
ATTY. edm/mjm
DUE DATE DKT. BY dkm
CALL UP DKT. BY
RESP. SENT DKT. BY
REVIEWED BY ATTY DATE
OK TO FILE DATE

3 mo. Response to O.A. due 11/29/00

PTO-90C (Rev. 2/95)

2 - Mail Copy

Office Action Summary	Application No. 09/382,622	Applicant(s) Dess et al.
	Examiner Gailene R. Gabel	Group Art Unit 1641

☒ Responsive to communication(s) filed on *Aug 25, 1999*

☐ This action is **FINAL.**

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire *3* month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claim

☒ Claim(s) *1-30, 51, and 52* is/are pending in the applicat

Of the above, claim(s) _____ is/are withdrawn from consideration

☐ Claim(s) _____ is/are allowed.

☒ Claim(s) *1-30, 51, and 52* is/are rejected.

☐ Claim(s) _____ is/are objected to.

☐ Claims _____ are subject to restriction or election requirement.

Application Papers

☒ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on _____ is ☐ approved ☐ disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☒ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) _____.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____

☒ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☒ Notice of References Cited, PTO-892

☒ Information Disclosure Statement(s), PTO-1449, Paper No(s). *3-5*

☐ Interview Summary, PTO-413

☒ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

— SEE OFFICE ACTION ON THE FOLLOWING PAGES —

Notice of References Cited

Application No.	Applicant(s)		
09/382,622	Dess et al.		
Examiner		Group Art Unit	Page 1 of 1
Gallene R. Gabel		1641	

U.S. PATENT DOCUMENTS

		DOCUMENT NO.	DATE	NAME	CLASS	SUBCLASS
	A	US 5,780,052	1/1998	Khaw et al.	424	450
	B	US 5,225,182	1/1993	Sharma	424	9
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS
	N						
	O						
	P						
	Q						
	R						
	S						
	T						

NON-PATENT DOCUMENTS

		DOCUMENT (Including Author, Title, Source, and Pertinent Pages)	DATE
	U	Serafini et al. Iodine-123-Rose Bengal: An improved hepatobiliary imaging agent, Journal of Nuclear Medicine, 16(7): 629-632 (1975)	1975
	V		
	W		
	X		

DETAILED ACTION

Priority

1. An application in which the benefits of an earlier application are desired must contain a specific reference to the prior application in the first sentence of the specification (37 CFR 1.78).

Drawings

2. The drawings in this application are objected to by the Draftsperson (see PTO-948 attached). Correction is required. However, formal correction of noted defect can be deferred until application is allowed by the examiner.

Specification

3. The abstract of the disclosure is objected to because it does not sufficiently describe the disclosure. Furthermore, the abstract of disclosure is replete with grammatical errors. Correction is required. See MPEP § 608.01(b). Applicant is reminded of the proper language and format for an abstract of the disclosure.

The abstract should be in narrative form and generally limited to a single paragraph on a separate sheet within the range of 50 to 250 words. The abstract should describe the disclosure sufficiently to assist readers in deciding whether there is a need for consulting the full patent text

for details. The language should be clear and concise and should not repeat information given in the title.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

4. Claims 2-30 and 52 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 2 recites improper Markush language in reciting "selected from the group comprising". See also claims 3, 8, 9, 11, 19, 22, 27, and 29.

Regarding claim 2, the phrase "and its derivatives" renders the claims indefinite because the claim includes elements not actually disclosed (those encompassed by "and its derivatives"), thereby rendering the scope of the claim unascertainable. See MPEP § 2173.05(d). See also claims 3 and 9.

Claim 4 recites improper and overlapping Markush language in reciting "selected from the group comprising ... or".

Regarding claim 4, the phrase " including those containing aldehydes, ketones, alcohols..." renders the claim indefinite because the claim includes elements not actually

disclosed (those encompassed by "including those containing"), thereby rendering the scope of the claim unascertainable. See MPEP § 2173.05(d).

Claim 4 is indefinite in reciting "DNA and RNA". Acronyms or abbreviations must be recited at least one time in a set of claims.

Regarding claim 4, the phrase "or other hydrophilic or hydrophobic moieties" renders the claim indefinite because the claim includes elements not actually disclosed (those encompassed by "or other"), thereby rendering the scope of the claim unascertainable. See MPEP § 2173.05(d).

Claim 6 is indefinite in reciting "CAT scan". Acronyms or abbreviations must be recited at least one time in a set of claims.

The term "large content" in claim 8 is a relative and subjective term that renders the claim indefinite. The term "large content" is not defined by the claim, the specification does not provide a standard for ascertaining their requisite degree, and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention.

Claim 11 is fails to recite positive limitation in reciting "said vehicle being selected from the group".

Claim 12 is rejected under 35 U.S.C. 112, second paragraph, as being incomplete for omitting essential structural cooperative relationships of elements, such omission amounting to a gap between the necessary structural connections. See MPEP § 2172.01. In this case, it is unclear what structural cooperative relationship exists between "diseased tissue" in claim 12 and

"biologically sensitive structures" and "cellular membranes" in the instant claim and all subsequent claims that make reference thereto. See claims 13-17, 21, 23-24, and 28. For example, is the "biologically sensitive structure" the same as or a part of "the diseased tissue".

Claim 12 is indefinite and unclear in reciting "a preference for" since the term "preference" is a subjective term which lacks comparative basis for defining its metes and bounds. See also claim 13.

Claim 12 is indefinite and unclear in reciting "biologically sensitive structures" since the term "biologically sensitive" is subjective and lacks comparative basis for defining its metes and bounds. See also claims 14, 15, 16, 17, 21, 23, 24, and 28.

Claims 14 and 15 are indefinite and unclear in reciting "biologically targets" and "chemically targets said biologically sensitive structures" because it is unclear how each mode of "targeting" is effected in relation to claims 16, 17, and 18.

Claims 16 and 17 lack antecedent support in reciting "said targeting".

Accordingly, claim 16 is confusing in reciting "said targeting is by chemical partitioning of the agent" because it is dependent upon claim 14 which recites "biologically targets...".

Regarding claim 19, the phrase "or its derivatives" renders the claims indefinite because the claim includes elements not actually disclosed (those encompassed by "or its derivatives"), thereby rendering the scope of the claim unascertainable. See MPEP § 2173.05(d).

Claim 21 lacks antecedent support in reciting "said targeting".

Claim 21 is indefinite and unclear in reciting "physically" since the term "physically" is a subjective term which lacks comparative basis for defining its metes and bounds. See also claims 22, 28, and 29.

Claim 23 lacks antecedent support in reciting "said targeting".

Claim 24 lacks antecedent support in reciting "said targeting".

Claim 28 lacks antecedent support in reciting "said targeting".

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

5. Claims 1-30 and 51-52 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for treatment of diseased tissue such as cancer and tumor cell tissues, does not reasonably provide enablement for treatment of any diseased tissue such as parasitized and infected muscle tissue. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

As set forth in *In re Wands,* 858 F .2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988), the factors to be considered in determining whether a claimed invention is enabled throughout its scope without undue experimentation include the quantity of the experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the

nature of the invention, the state of prior art, the relative skill of those in the art, and the breadth of the claims.

As to the diseased tissue, the direction and guidance in the specification is notably limited to *specific* neoplastic tissue, such as cancer tissue and tumors. While this is sufficient guidance and direction for treatment of neoplastic tissue, wherein radiosensitizer agents are introduced and concentrate preferentially into such tissue to allow increased dose of ionizing radiation for treatment, it does not teach or suggest how to treat alternative diseased tissue such as infected tissue and pathologically enlarged cardiac muscle tissue, etc. Based on this limited disclosure and direction, one of the skill in the art would not know how to detect alternative diseased tissue, using the instant halogenated xanthene radiosensitizer agent, without undue experimentation.

Claim Rejections - 35 USC § 102

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

6. Claims 1-3, 5-8, 10, 12-13, 15-16, 23, 28-30, and 51 are rejected under 35 U.S.C. 102(b) as being inherently anticipated by Serafini et al. (Journal of Nuclear Medicine, 1975).

Serafini et al. teach a radiosensitizer agent for use in treating diseased tissue (radiopharmaceutical agent). Specifically, Serafini et al. teach halogenated or iodinated rose bengal which is principally tetrachlorotetraiodofluorescein, that allows for rapid and efficient incorporation into molecules resulting to overall reduction in imaging time and radiation

exposure together with improved images (see Abstract). In a study, Serafini et al. intravenously injected iodinated Rose Bengal into healthy volunteers, then blood clearance studies were performed in addition to urinary clearance and simultaneous and sequential scintiphotos were taken of the cardiac, liver, biliary, and intestinal systems. Sufficient concentration into diseased tissue (localization) within the liver then the rest of the biliary tree of the radiopharmaceutical agent is observed with marked improvement in anatomic detail showing specific areas of radioactive concentrations (see page 630, column 2).

7. Claims 1-3, 5-10, 12-13, 15-16, 23, 28-30, and 51 are rejected under 35 U.S.C. 102(b) as being inherently anticipated by Neckers D. (Journal of Photochemistry and Photobiology, A: Chemistry 47: 1-29 (1989)).

Neckers teaches a radiosensitizer agent: Rose Bengal or 2,4,5,7- tetraiodo-3', 4', 5', 6'-tetrachlorofluorescein, which has the spectral, photochemical, and photophysical properties of a halogenated xanthene. Neckers teaches that Rose Bengal, disodium salt is characterized by (1) large absorption in all solvents, (2) capacity as imaging agent (shows fluorescence), (3) its triplet is completely quenched by oxygen, (4) concentration on selected tissues, i.e. tumor (its spectrum is most diagnostic of its immediate environment), (5) it bleaches in protic, polar solvents, (6) is a photodynamic sensitizer, (7) its singlet may be quenched by strong oxidizing agents (see page 1). The absorption and emission spectra of known Rose Bengal derivatives are enumerated in Table 2 and 3, respectively. In conclusion, Neckers teaches the halogenated xanthene, Rose Bengal,

whose characteristic increased absorptivity, fluorescence, and specific diagnostic concentration on specific spectrum provides inherent capability to be sensitized or activated using X-rays and other ionizing radiation in order to treat diseased tissue, in addition to its current use as imaging agent when exposed to such ionizing radiation.

8. Claims 12-17, 21-24, 28-29 and 52 are rejected under 35 U.S.C. 102(b) as being anticipated by Norman et al., Invest Radiol, 26: S120-S121 (1991).

Norman et al. teach a radiosensitizer agent (iodinated contrast media/ gandolinium) for treatment of diseased tissue using radiosensitization or ionizing radiation wherein doses absorbed from diagnostic X-rays are enhanced. Norman et al. specifically teach that the radiosensitizer agent exhibits preference to (help localize) biologically sensitive diseased tumor tissues. In experimentation, Norman et al. showed that survival of rabbits increased when irradiation therapy was preceded by injection (infusion) of the iodinated contrast media (see page S120, column 1, paragraph 1). Norman et al. further teach that the dose enhancement factor (DEF) which increases linearly with the concentration of iodine can also be achieved with conventional administration of contrast media (S120, column 1 and 2). Figure 1 shows a plot of the DEF as a function of the iodine concentration in a lymphocyte medium during irradiation at 140 kVp. In conclusion, the therapeutic ratio, the ratio of radiation dose absorbed by a diseased brain tumor tissue versus that absorbed by the surrounding normal brain tissues increases with increasing iodinated contrast media in the diseased tissue.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

9. Claims 18 and 25 are rejected under 35 U.S.C. 103(a) as being unpatentable over Norman et al. (Invest Radiol, 26: S120-S121, 1991) in view of Khaw et al. (US 5,780,052).

Norman et al. have been discussed supra. Norman et al. fail to teach encapsulating the radiosensitizer agent for delivery into diseased cancer tumor.

Khaw et al. disclose a method of enhancing effects of therapy that kills diseased (malignant/tumor) cells in vivo by providing (immuno)liposomes specific for an internal cellular antigen present in degenerating neoplastic cells. Techniques are known for liposome targeting such as conjugating antibodies to cell-surface (malignant) antigens to pharmacologically active agents and labels to permit diagnosis, localization, and therapy toward tumors (see column 7, line 48 to column 8, line 3). The liposomes contain antineoplastic agent for initiating therapy in a mammal to kill malignant cells in vivo (see column 2, last paragraph). The antineoplastic agents include radiosensitizing agents, cytotoxic agents, and radionuclides (see column 3, first paragraph and column 4, lines 18-27). In diagnostic procedures, (immuno)liposomes containing radiosensitizer and diagnostic agent which are specific for intracellular antigens, i.e. a detectable molecule such as an imaging agent are injected into a patient receiving radiation therapy.

Following administration, an imaging technique is employed such as computed axial tomography (CAT) scan and X-ray imaging (see column 16, line 18 to column 17, lines 3).

It would have been obvious to one of ordinary skill in the art at the time of the invention to administer the radiosensitizer agent taught by Norman using liposomal targeting mode of infusion as taught by Khaw because Khaw specifically taught that antineoplastic agents such as radiosensitizer agents can be incorporated into liposomes for use in initiating therapy in animals to kill malignant tumors in vivo and such liposome targeting methods containing pharmacologically active agents and labels allow diagnosis, localization, and therapy directly toward specific tissues. One of ordinary skill in the art at the time of the instant invention would have been motivated to incorporate the teachings of Norman into the method of Khaw because of the multifunctional capacity as achieved in combining treatment and imaging capability using a single radiosensitization agent for treatment of cancer or tumorous tissues.

10. Claims 4, 11, 14, 17-22, and 24-27 are rejected under 35 U.S.C. 103(a) as being unpatentable over Serafini et al. (Journal of Nuclear Medicine, 1975) or Neckers D. (Journal of Photochemistry and Photobiology, A: Chemistry 47: 1-29 (1989)) in view of Khaw et al. (US 5,780,052).

Serafini et al. and Neckers have been discussed supra.

Serafini et al. and Neckers fail to teach encapsulating the halogenated xanthene, Rose Bengal, for delivery into diseased cancer tumor.

Khaw et al. has been discussed supra.

It would have been obvious to one of ordinary skill in the art at the time of the invention to administer halogenated xanthene taught by Serafini et al. or Neckers using liposomal targeting mode of infusion as taught by Khaw because Khaw specifically taught that antineoplastic agents such as halogenated xanthenes can be incorporated into liposomes for use in initiating therapy in animals to kill malignant tumors in vivo and such liposome targeting methods containing pharmacologically active agents and labels allow diagnosis, localization, and therapy directly toward specific tissues. One of ordinary skill in the art at the time of the instant invention would have been motivated to incorporate the teachings of Serafini or Neckers into the method of Khaw because of the multifunctional capacity as achieved in combining treatment and imaging capability using a single radiosensitization agent for treatment of cancer or tumorous tissues.

11. No claims are allowed.

Remarks

12. The prior art made of record and not relied upon is considered pertinent to applicant's disclosure.

Sharma discloses a method for vectored delivery of physiologically active chemical agents to target organ, tissue, or cell of interest, including rose bengal as a vectoring agent.

13. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Gail Gabel whose telephone number is (703) 305-0807. The examiner can normally be reached on Monday to Thursday from 7:00 AM to 4:30 PM. The examiner can also be reached on alternate Fridays from 7:00 AM to 3:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Long Le, can be reached on (703) 308-3399. The fax phone number for the organization where this application or proceeding is assigned is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-0196.

8/24/00

Gail Gabel
Patent Examiner
Group 1641

LONG V. LE
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

LIST OF PUBLICATIONS/MATERIALS CITED BY APPLICANT	Attorney Docket No. PHO-107 DIV	Serial No. Not Assigned
	Applicant H. Craig DEES et al	
	Filing Date Herewith	Group unknown

1504 U.S. PTO 09/382622 08/25/99

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE
	3,868,950	03/04/75	Kato	128	066	11/08/73
	3,986,513	10/19/76	Stuhl	128	373	01/29/76
	4,066,650	01/03/78	Egyud	260	281.G	02/25/75
	4,172,979	10/30/79	Morrison	250	505	06/15/78
	4,241,060	12/23/80	Smithen	424	248.57	08/03/78
	4,282,232	08/04/81	Agrawal	424	267	04/26/79
	4,371,540	02/01/83	Lee et al	424	273.R	08/22/80
	4,444,189	04/24/84	Seiverd	128	395	12/21/81
	4,462,992	07/31/84	Agrawal et al	424	180	02/08/82
	4,652,562	03/24/87	Berenyi nee Poldermann	514	237	06/04/86
	4,681,091	07/21/87	Picker et al	128	001.R	11/27/85
	4,691,332	09/01/87	Burstein et al	378	007	03/14/84

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB-CLASS	FILING DATE

OTHER PUBLICATIONS/MATERIALS
(Including Author, Title, Date, Pertinent Pages)

EXAMINER: Andrew B. Gabel	DATE CONSIDERED: 8-17-00

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP form. Draw line through citation if not in conformance and not considered. Include a copy of this form with the next communication to applicant.

LIST OF PUBLICATIONS/MATERIALS CITED BY APPLICANT	Attorney Docket No. PHO-107 DIV	Serial No. Not Assigned
	Applicant H. Craig DEES et al.	
	Filing Date Herewith	Group unknown

jc584 U.S. PTO 09/382922 08/25/99

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE
	4,820,258	04/11/89	Mondain-Monval	600	001	03/24/87
	4,856,528	08/15/89	Yang et al	128	653	06/26/87
	4,880,821	11/14/89	Saari	514	331	08/17/88
	4,897,423	01/30/90	Saari et al	514	603	08/02/88
	4,921,963	05/01/90	Skov et al	548	101	04/13/87
	4,927,941	05/22/90	Kagiya et al	548	264.8	06/09/88
	4,945,102	07/31/90	Suzuki et al	514	398	09/21/88
	4,954,515	09/04/90	Suto	514	398	11/25/88
	4,977,273	12/11/90	Kagiya et al	548	339	12/12/89
	5,008,907	04/16/91	Norman et al	378	065	05/31/89
	5,026,694	06/25/91	Skov et al	514	184	06/30/89
	5,036,089	07/30/91	Suto	514	376	06/04/90
	5,036,096	07/30/91	Suto	514	398	06/04/90

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	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB-CLASS	FILING DATE

OTHER PUBLICATIONS/MATERIALS
(Including Author, Title, Date, Pertinent Pages)

	1. Bezman, S.A. et al, "Photodynamic Inactivation of E. Coli by Rose Bengal Immobilized on Polystyrene Beads," Biochemistry and Photobiology, vol. 28, pp. 325-329, 1978.

EXAMINER: Darlene R. Dahl

DATE CONSIDERED: 8-17-00

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP form. Draw line through citation if not in conformance and not considered. Include a copy of this form with the next communication to applicant.

LIST OF PUBLICATIONS/MATERIALS CITED BY APPLICANT	Attorney Docket No. PHO-107 DIV	Serial No. Not Assigned
	Applicant H. Craig DEES et al.	
	Filing Date Herewith	Group unknown

jc584 U.S. PTO 09/382622 08/25/99

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE
	5,053,006	10/01/91	Watson	604	52	04/02/90
	5,064,849	11/12/91	Suzuki et al	514	383	04/18/90
	5,147,652	09/15/92	Egyud	424	450	07/03/90
	5,175,287	12/29/92	Lee et al	544	183	09/18/89
	5,215,738	06/01/93	Lee et al	424	010	06/01/90
	5,270,330	12/14/93	Suzuki et al	514	398	07/24/92
	5,294,715	03/15/94	Papadopoulou-Rosenzweig et al	546	106	02/01/91
	5,302,369	04/12/94	Day et al	424	001.29	08/29/91
	5,304,654	04/19/94	Kagiya et al	548	327.5	09/09/91
	5,342,959	08/30/94	Beylin et al	548	327.5	08/05/93
	5,457,183	10/10/95	Sessler et al	534	011	10/12/93
	5,481,000	01/02/96	Beylin et al	548	229	12/19/94
	5,543,527	08/06/96	Beylin et al	548	110	05/18/95
	5,591,422	01/07/97	Hemmi et al	424	009.36	06/06/95
	5,599,923	02/04/97	Sessler et al	540	145	02/15/94
	5,601,802	02/11/97	Hemmi et al	424	009.36	06/07/95

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	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB-CLASS	FILING DATE

OTHER PUBLICATIONS/MATERIALS
(Including Author, Title, Date, Pertinent Pages)

	2. Fluhler, E.N. et al, "Laser Intensity and Wavelength Dependence of Rose Bengal Photosensitized Inhibition of Red Blood Cell Acetylcholinesterase," Biochimica et Biophysica Acta, 990, pp. 269-275, 1989.

EXAMINER: Andrew R. Dalal

DATE CONSIDERED: 8-17-00

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP form. Draw line through citation if not in conformance and not considered. Include a copy of this form with the next communication to applicant.

LIST OF PUBLICATIONS/MATERIALS CITED BY APPLICANT	Attorney Docket No. PHO-107 DIV	Serial No. Not Assigned
	Applicant H. Craig DEES et al.	
	Filing Date Herewith	Group unknown

JC584 U.S. PTO 09/382622 08/25/99

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE
	5,602,142	02/11/97	Papadopolou-Rosenzweig	514	290	12/21/94
	5,616,584	04/01/97	Lee et al	514	243	01/26/95
	5,622,946	04/22/97	Sessler et al	514	185	05/10/95
	5,624,925	04/29/97	Lee et al	514	243	01/26/95
	5,641,764	06/24/97	Martin et al	514	080	05/15/95
	5,654,423	08/05/97	Kahl et al	540	145	05/23/95
	5,659,048	08/19/97	Beylin et al	548	327.5	05/23/95
	5,700,825	12/23/97	Hofer et al	514	397	03/31/95
	5,780,653	07/14/98	Tao et al	549	510	06/07/95
	5,829,448	11/03/98	Fisher et al	128	898	10/30/96
	5,832,931	11/10/98	Wachter et al	128	898	10/30/96

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	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB-CLASS	FILING DATE

OTHER PUBLICATIONS/MATERIALS
(Including Author, Title, Date, Pertinent Pages)

	3. Huang, A.J.W. et al, "Photothrombosis of Corneal Neovascularization by Intravenous Rose Bengal and Argon Laser Irradiation," Arch Ophthalmol, vol. 106, pp. 680-685, May, 1988. Neckers, D.C., "Rose Bengal," Journal of Photochemistry and Photobiology, A. Chemistry, vol. 47, pp. 1-29, 1989.

EXAMINER: Smilene R. Graham	DATE CONSIDERED: 8-17-01

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP form. Draw line through citation if not in conformance and not considered. Include a copy of this form with the next communication to applicant.

LIST OF PUBLICATIONS/MATERIALS CITED BY APPLICANT	Attorney Docket No. PHO-107 D1 ✓	Serial No. Not Assigned
	Applicant H. Craig DEES et al.	
	Filing Date Herewith	Group unknown

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB-CLASS	FILING DATE

OTHER PUBLICATIONS/MATERIALS
(Including Author, Title, Date, Pertinent Pages)

SH	4. Norman, A. et al, "X-Ray Phototherapy for Solid Tumors," Acad Radiol, vol. 5, (suppl 1) pp. s177-179, April, 1998.
SH	5. Norman, A. et al, "Point/Counterpoint: Radiation Doses in Radiation Therapy are not Safe," Med. Phys. vol. 24 (11), pp. 1710-1713, November, 1997.
SH	6. Matsudaira, H. et al, "Iodine Contrast Medium Sensitizes Cultured Mammalian Cells to X Rays but not to Gamma Rays," Radiation Research, vol. 84, pp. 144-148, 1980.
SH	7. Norman, A. et al, "Iodinated Contrast Agents for Brain Tumor Localization and Radiation Dose Enhancement," Invest. Radiol, vol. 26, pp. s120-1221, 1991.
SH	8. Rubin, D. et al, "Nanoparticulate Contrast Media, Blood-Pool and Liver-Spleen Imaging," Investigative Radiology, vol. 29, suppl. 2, pp. s280-283, 1994.

EXAMINER: Darlene R. Gabel	DATE CONSIDERED: 8-17-01

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP form. Draw line through citation if not in conformance and not considered. Include a copy of this form with the next communication to applicant.

LIST OF PUBLICATIONS/MATERIALS CITED BY APPLICANT	Attorney Docket No. PHO-107 DIV	Serial No. Not Assigned
	Applicant H. Craig DEES et al.	
	Filing Date Herewith	Group unknown

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB-CLASS	FILING DATE

OTHER PUBLICATIONS/MATERIALS
(Including Author, Title, Date, Pertinent Pages)

SH	9. Budavari, S., ed. et al, The Merck Index, Merck & Co., Inc., 11th ed., p. 4943, 1989.
SH	10. Iwamoto, K.S. et al, "Radiation Dose Enhancement Therapy with Iodine in Rabbit VX-2 Brain Tumors," Radiation Therapy and Oncology, vol. 8, pp. 161-170, 1987.
SH	11. Miller, R.W. et al, "Evaluation of Incorporated Iododeoxyuridine Cellular Radiosensitization by Photon Activation Therapy," Int. J. Radiation Oncology, Biol. Phys., vol. 13, pp. 1193-1197, 1987.
SH	12. Zajusz, A. et al, "Normobaric Oxygen as a Sensitizer in Radiotherapy for Advanced Head and Neck Cancer," Neoplasma, vol. 42, number 3, pp. 137-140, 1995.
SH	13. United States Statutory Invention Registration No. H505 to Slatkin et al, for BORON UPTAKE IN TUMORS, CEREBRUM AND BLOOD FROM [^{10}B]$NA_4B_{24}H_{22}S_2$, published August 2, 1988.

EXAMINER: Darlene R. Gabel	DATE CONSIDERED: 8-17-00

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP form. Draw line through citation if not in conformance and not considered. Include a copy of this form with the next communication to applicant.

LIST OF PUBLICATIONS CITED BY APPLICANT	Attorney Docket No. PHO 107 DIV	Serial No. 09/382,622
	Applicant H. Craig DEES et al	RECEIVED
	Filing Date August 25, 1999	Group DEC 08 1999 Group 3700

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE
SV	5,008,907	04/16/91	Norman et al	378	65	05/31/89
SV	5,019,368	05/28/91	Epstein et al	424	1.1	02/23/89
SV	5,567,765	10/22/96	Moore et al	524	801	02/23/96
SV	5,676,928	10/14/97	Klaveness et al	424	9.321	06/06/95
SV	5,733,572	03/31/98	Unger et al	424	450	11/29/94
SV	5,807,231	09/15/98	Liprie	600	3	10/31/95

OIPE DEC X 6 1999 PATENT & TRADEMARK OFFICE

FOREIGN PATENT DOCUMENT

	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE

OTHER PUBLICATIONS
(Including Author, Title, Date, Pertinent Pages)

SV	Mello, R.S. et al, "Radiation Dose Enhancement in Tumors with Iodine," *Medical Physics*, vol. 1, no. 1, pp. 75-78, Jan/Feb 1983. Norman, A. et al, "X-Ray Phototherapy for Canine Brain Masses," *Radiation Oncology Investigations*, vol. 5, pp. 8-14, 1997.

EXAMINER: Darlene R. Dahl	DATE CONSIDERED: 8-17-00

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP form. Draw line through citation if not in conformance and not considered. Include a copy of this form with the next communication to applicant.

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on December 2, 1999

Cristine M. Noll

Date 12/2/99

RECEIVED DEC 08 1999 Group 3700

Please type a plus sign (+) inside this b[redacted] +

PTO/SB/08B (10-96)
Patent and Trad[redacted] d for use through 10/31/99. OMB 0651-0031
Office: U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of informatio... unless it contains a valid OMB control number.

Substitute for form 1449B/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	09/382,622
Filing Date	08/25/1999
First Named Inventor	DEES et al.
Group Art Unit	
Examiner Name	
Attorney Docket Number	PHO 107 DIV

OTHER PRIOR ART – NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
[illegible]		SERAFINI ET AL, IOdine-123-Rose Bengal: An Improved Hepatobiliary Imaging Agent, Journal of Nuclear Medicine, 1990.	

OIPE JC03
MAR 27 2000
PATENT & TRADEMARK OFFICE

Examiner Signature	[illegible]	Date Considered	8-17-00

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] Unique citation designation number. [2] Applicant is to place a check mark here if English language Translation is attached.

Burden Hour Statement: This form is estimated to take 2.0 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

Form PTO 948 (Rev. 8-98) U.S. DEPARTMENT OF COMMERCE - Patent and Trademark Office Application No. 29/102,222

NOTICE OF DRAFTSPERSON'S PATENT DRAWING REVIEW

The drawing(s) filed (insert date) 8/25/99 are:

A. ☐ approved by the Draftsperson under 37 CFR 1.84 or 1.152.

B. ☒ objected to by the Draftsperson under 37 CFR 1.84 or 1.152 for the reasons indicated below. The Examiner will require submission of new, corrected drawings when necessary. Corrected drawing must be sumitted according to the instructions on the back of this notice.

1. DRAWINGS. 37 CFR 1.84(a): Acceptable categories of drawings: Black ink. Color.
 - ___ Color drawings are not acceptable until petiton is granted. Fig(s) _____
 - ___ Pencil and non black ink not permitted. Fig(s) _____
2. PHOTOGRAPHS. 37 CFR 1.84 (b)
 - ___ 1 full-tone set is required. Fig(s) _____
 - ___ Photographs not properly mounted (must use brystol board or photographic double-weight paper). Fig(s) _____
 - ___ Poor quality (half-tone). Fig(s) _____
3. TYPE OF PAPER. 37 CFR 1.84(e)
 - ___ Paper not flexible, strong, white, and durable. Fig(s) _____
 - X Erasures, alterations, overwritings, interlineations, folds, copy machine marks not accepted. Fig(s) 1-4
 - ___ Mylar, ~~velum paper is~~ not acceptable (too thin). Fig(s) _____
4. SIZE OF PAPER. 37 CFR 1.84(f): ~~Acceptable sizes:~~
 - ___ 21.0 cm by 29.7 cm (DIN size A4)
 - ___ 21.6 cm by 27.9 cm (8 1/2 x 11 inches)
 - ___ All drawing sheets not the same size. Sheet(s) _____
 - ___ Drawings sheets not an acceptable size. Fig(s) _____
5. MARGINS. 37 CFR 1.84(g): Acceptable margins:

 Top 2.5 cm Left 2.5cm Right 1.5 cm Bottom 1.0 cm
 SIZE: A4 Size

 Top 2.5 cm Left 2.5 cm Right 1.5 cm Bottom 1.0 cm
 SIZE: 8 1/2 x 11

 Margins not acceptable. Fig(s) 2-3

 _____ Top (T) X Left (L)

 _____ Right (R) _____ Bottom (B)
6. VIEWS. 37 CFR 1.84(h)

 REMINDER: Specification may require revision to correspond to drawing changes.

 Partial views. 37 CFR 1.84(h)(2)
 - ___ Brackets needed to show figure as one entity. Fig(s) _____
 - ___ Views not labeled separately or properly. Fig(s) _____
 - ___ Enlarged view not labeled separetely or properly. Fig(s) _____
7. SECTIONAL VIEWS. 37 CFR 1.84 (h)(3)
 - ___ Hatching not indicated for sectional portions of an object. Fig(s) _____
 - ___ Sectional designation should be noted with Arabic or Roman numbers. Fig(s) _____
8. ARRANGEMENT OF VIEWS. 37 CFR 1.84(i)
 - ___ Words do not appear on a horizontal, left-to-right fashion when page is either upright or turned so that the top becomes the right side, except for graphs. Fig(s) _____
9. SCALE. 37 CFR 1.84(k)
 - ___ Scale not large enough to show mechanism without crowding when drawing is reduced in size to two-thirds in reproduction. Fig(s) _____
10. CHARACTER OF LINES, NUMBERS, & LETTERS. 37 CFR 1.84(i)
 - ___ Lines, numbers & letters not uniformly thick and well defined, clean, durable, and black (poor line quality). Fig(s) _____
11. SHADING. 37 CFR 1.84(m)
 - ___ Solid black areas pale. Fig(s) _____
 - X Solid black shading not permitted. Fig(s) 2-3
 - ___ Shade lines, pale, rough and blurred. Fig(s) _____
12. NUMBERS, LETTERS, & REFERENCE CHARACTERS. 37 CFR 1.84(p)
 - ___ Numbers and reference characters not plain and legible. Fig(s) _____
 - ___ Figure legends are poor. Fig(s) _____
 - ___ Numbers and reference characters not oriented in the same direction as the view. 37 CFR 1.84(p)(1) Fig(s) _____
 - ___ English alphabet not used. 37 CFR 1.84(p)(2) Figs _____
 - ___ Numbers, letters and reference characters must be at least .32 cm (1/8 inch) in height. 37 CFR 1.84(p)(3) Fig(s) _____
13. LEAD LINES. 37 CFR 1.84(q)
 - ___ Lead lines cross each other. Fig(s) _____
 - ___ Lead lines missing. Fig(s) _____
14. NUMBERING OF SHEETS OF DRAWINGS. 37 CFR 1.84(t)
 - ___ Sheets not numbered consecutively, and in Arabic numerals beginning with number 1. Sheet(s) _____
15. NUMBERING OF VIEWS. 37 CFR 1.84(u)
 - ___ Views not numbered consecutively, and in Arabic numerals, beginning with number 1. Fig(s) _____
16. CORRECTIONS. 37 CFR 1.84(w)
 - ___ Corrections not made from prior PTO-948 dated _____
17. DESIGN DRAWINGS. 37 CFR 1.152
 - ___ Surface shading shown not appropriate. Fig(s) _____
 - ___ Solid black shading not used for color contrast. Fig(s) _____

COMMENTS

REVIEWER [illegible] DATE 1/5/00 TELEPHONE NO. 703 305-8227

ATTACHMENT TO PAPER NO. _____

INFORMATION ON HOW TO EFFECT DRAWING CHANGES

1. Correction of Informalities--37 CFR 1.85

File new drawings with the changes incorporated therein. The application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application, should be placed on the back of each sheet of drawings in accordance with 37 CFR 1.84(c). Applicant may delay filing of the new drawings until receipt of the Notice of Allowability (PTOL-37). Extensions of time may be obtained under the provisions of 37 CFR 1.136. The drawing should be filed as a separate paper with a transmittal letter addressed to the Drawing Processing Branch.

2. Timing for Corrections

Applicant is required to submit **acceptable** corrected drawings within the three-month shortened statutory period set in the Notice of Allowability (PTOL-37). If a correction is determined to be unacceptable by the Office, applicant must arrange to have acceptable corrections resubmitted within the original three-month period to avoid the necessity of obtaining an extension of time and paying the extension fee. Therefore, applicant should file corrected drawings as soon a possible.

Failure to take corrective action within set (or extended) period will result in **ABANDONMENT** of the Application.

3. Corrections other than Informalities Noted by the Drawing Review Branch on the Form PTO-948

All changes to the drawings, other than informalities noted by the Drawing Review Branch, **MUST** be approved by the examiner before the application will be allowed. No changes will be permitted to be made, other than correction of informalities, unless the examiner has approved the proposed changes.

Exhibit B

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
09/382,622	08/25/99	DEES H	PHO-107-DIV

HM22/0227

EDWARD D MANZO
COOK ALEX MCFARRON MANZO CUMMINGS
& MEHLER LTD
200 WEST ADAMS STREET SUITE 2850
CHICAGO IL 60606

EXAMINER	
GABEL, G	
ART UNIT	**PAPER NUMBER**
1641	11

DATE MAILED: 02/27/01

RECEIVED
MAR 05 2001
COOK, ALEX, McFARRON,

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Photogen
CASE PHO-107DIV
ATTY: edm/mjm
DUE DATE DKT. BY time
CALL UP DKT. BY
RESP. SENT DKT. BY
REVIEWED BY ATTY. DATE
OK TO FILE DATE
3 mo. Response to FINAL O.A. due 4/27/01

Office Action Summary	Application No.	Applicant(s)	
	09/382,622	DEES ET AL.	
	Examiner	Art Unit	
	Gailene R. Gabel	1641	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136 (a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).

Status

1)☒ Responsive to communication(s) filed on *14 December 2000* .

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-10,12-16,18-20,23,25-27,50-52 and 54-59* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-10,12-16,18-20,23,25-27,50-52 and 54-59* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claims _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

a)☐ All b)☐ Some * c)☐ None of the CERTIFIED copies of the priority documents have been:

1.☐ received.

2.☐ received in Application No. (Series Code / Serial Number) _____ .

3.☐ received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. & 119(e).

Attachment(s)

14) ☐ Notice of References Cited (PTO-892)
15) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
16) ☒ Information Disclosure Statement(s) (PTO-1449) Paper No(s) *9,10* .
17) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
18) ☐ Notice of Informal Patent Application (PTO-152)
19) ☐ Other:

DETAILED ACTION

Amendment Entry

1. Applicant's amendment and response filed 12/4/00 in Paper No. 8 is acknowledged and has been entered. Claims 1-10, 12-16, 18-20, 23, 25-27, and 51-52 have been amended. Claims 11, 17, 21-22, 24, and 28-30 have been cancelled. Claims 54-59 have been added. Accordingly, claims 1-10, 12-16, 18-20, 23, 25-27, 50-52, and 54-59 are pending and under examination.

Priority

2. An application in which the benefits of an earlier application are desired must contain a specific reference to the prior application in the first sentence of the specification (37 CFR 1.78) including the status of the parent application.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

3. The rejection of claims 11, 17, 21-22, 24, and 28-30 under 35 U.S.C. 112, second paragraph, is now moot in light of Applicant's cancellation of the claims.

4. Claims 15-16, 23, 25-27, and 54 stand rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 15 has improper antecedent support in reciting "to biologically sensitive structures". Change to "to the to biologically sensitive structures" for proper antecedent basis.

Claim 16 has improper antecedent support in reciting "into biologically sensitive structures". Change to "into the to biologically sensitive structures" for proper antecedent basis.

Claim 16 lacks clear, distinct, sufficient, and consistent antecedent support in reciting "said targeting". Change to "said biological targeting" for proper, sufficient and consistent antecedent support.

Claim 23 has improper antecedent support in reciting "into biologically sensitive structures". Change to "into the to biologically sensitive structures" for proper antecedent basis.

Claim 23 lacks clear, distinct, sufficient, and consistent antecedent support in reciting "said targeting". Change to "said chemical targeting" for proper, sufficient and consistent antecedent support.

Claim 54 is vague and indefinite in reciting "at least one moiety at positions R^1 and R^{2}" because claim 12 to which it depends from does not recite a structure to make reference to. Accordingly, claim 54 lacks antecedent support.

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

5. The rejection of claims 11, 17, 21-22, 24, and 28-30 under 35 U.S.C. 112, first paragraph, is now moot in light of Applicant's cancellation of the claims.

6. In light of Applicant's amendment and arguments, the rejection of claims 1-10, 12-16, 18-20, 23, 25-27, and 50-52 under 35 U.S.C. 112, first paragraph, is hereby, withdrawn.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless -
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

7. The rejection of claims 11, 17, 21-22, 24, and 28-30 under 35 U.S.C. 102(b), is now moot in light of Applicant's cancellation of the claims.

8. Claims 1-3, 5-8, and 12-13 stand rejected under 35 U.S.C. 102(b) as being inherently anticipated by Serafini et al. (Journal of Nuclear Medicine, 1975) for reasons of record.

Additionally, new claims 58-59 are rejected under 35 U.S.C. 102(b) as being inherently anticipated by Serafini et al. (Journal of Nuclear Medicine, 1975).

Serafini et al. has been discussed in Paper No. 7.

9. In light of Applicant's amendment and arguments, the rejection of claims 10, 15-16, 23, and 51 under 35 U.S.C. 102(b) as being anticipated by Serafini et al. (Journal of Nuclear Medicine, 1975) is, hereby, withdrawn.

10. Claims 1-3, 5-9, and 12-13 stand rejected under 35 U.S.C. 102(b) as being inherently anticipated by **Neckers D.** (Journal of Photochemistry and Photobiology, A: Chemistry 47: 1-29 (1989)) for reasons of record.

Additionally, new claims 58-59 are rejected under 35 U.S.C. 102(b) as being inherently anticipated by **Neckers D.** (Journal of Photochemistry and Photobiology, A: Chemistry 47: 1-29 (1989)).

Neckers et al. has discussed in Paper No. 7.

11. In light of Applicant's amendment and arguments, the rejection of claims 10, 15-16, 23, and 51 under 35 U.S.C. 102(b) as being anticipated by Neckers D. (Journal of Photochemistry and Photobiology, A: Chemistry 47: 1-29 (1989)) is, hereby, withdrawn.

12. In light of Applicant's amendment and arguments, the rejection of claims 12-16, 23, and 52 under 35 U.S.C. 102(b) as being anticipated by Norman et al., Invest Radiol, 26: S120-S121 (1991) is, hereby, withdrawn.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

13. The rejection of claims 11, 17, 21-22, and 24 under 35 U.S.C. 103(a), is now moot in light of Applicant's cancellation of the claims.

14. In light of Applicant's amendment and arguments, the rejection of claims 18 and 25 under 35 U.S.C. 103(a) as being unpatentable over Norman et al. (Invest Radiol, 26: S120-S121, 1991) in view of Khaw et al. (US 5,780,052) is, hereby, withdrawn.

15. Claims 4, 14, and 18-20, 25-27 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Serafini et al. (Journal of Nuclear Medicine, 1975) or Neckers D. (Journal of Photochemistry and Photobiology, A: Chemistry 47: 1-29 (1989)) in view of Khaw et al. (US 5,780,052) for reasons of record.

Additionally, claims 15-16, 23, and new claims 54-55 are rejected under 35 U.S.C. 103(a) as being unpatentable over Serafini et al. (Journal of Nuclear Medicine, 1975) or Neckers D. (Journal of Photochemistry and Photobiology, A: Chemistry 47: 1-29 (1989)) in view of Khaw et al. (US 5,780,052).

Serafini et al., Neckers and Khaw et al. have been discussed in Paper No. 7.

New Grounds of Rejection

16. Claim 10, 51, 52, and new claims 56-57 are rejected under 35 U.S.C. 103(a) as being unpatentable over Serafini et al. (Journal of Nuclear Medicine, 1975) or Neckers D. (Journal of Photochemistry and Photobiology, A: Chemistry 47: 1-29 (1989)) in view of Norman et al. (Invest Radiol, 26: S120-S121, 1991).

Serafini et al. and Neckers have been discussed in Paper No. 7. Serafini et al. and Neckers fail to teach activating the radiosensitizer agents with ionizing radiation at greater than or equal to 1 keV and less than or equal to 1000 MeV.

Norman et al. has been discussed in Paper No. 7.

Specifically, Norman et al. teach a radiosensitizer agent such as iodinated contrast media or gandolinium for treatment of diseased tissue using ionizing radiation wherein doses absorbed from diagnostic X-rays are enhanced. Norman et al. specifically teach that the radiosensitizer agent exhibits preference to biologically sensitive diseased tumor tissues. Figure 1 shows a plot of the DEF as a function of the iodine concentration in a lymphocyte medium during irradiation at 140 kVp.

It would have been obvious to one of ordinary skill in the art at the time of the instant invention to substitute Rose Bengal taught by Serafini and Neckers for the iodinated contrast agent taught by Norman because Serafini and Neckers specifically taught application of Rose Bengal into specific tissue for use as contrast or imaging agents and Norman specifically taught that halogenation or iodination of contrast media such as gandolinium or Rose Bengal such as taught by Serafini or Neckers can be activated by ionizing radiation for concentration and treatment of diseased tissue. One or ordinary skill in the art at the time of the instant invention would have been motivated to substitute the contrast media taught by Norman with the Rose Bengal of Serafini and Neckers because of the comparable and equivalent inherent characteristic preference of Rose Bengal towards biologically sensitive diseased tumor tissues that is, likewise, exhibited resulting from halogenation or iodination upon exposure with ionizing radiation.

Response to Arguments

17. Applicant's arguments filed 12/4/00 have been fully considered but they are not persuasive.

a) Applicant argues that Serafini et al. does not teach or suggest the agent of the claimed invention as a radiosensitizer agent for use with radiosensitization or ionizing radiation in the treatment of cancer or tumors.

In response, Serafini et al., indeed, teach rose bengal, a halogenated or iodinated xanthene, which is principally tetrachlorotetraiodofluorescein, which has an inherent capacity to rapidly and efficiently concentrate into cellular molecules upon exposure to ionizing radiation; thus, a radiosensitizer or a pharmaceutical agent which in combination with ionizing radiation, finds use in treating diseased tissue with overall reduction in radiation exposure to treat diseases such as cancer or tumor. Specifically, Applicant claims a radiosensitizer agent, halogenated xanthene, a known product clearly taught by Serafini et al. and which has the inherent property to concentrate into diseased tissue (as shown by Serafini et al.). Therefore, the product of the present invention is concluded to be inherently anticipated by Serafini et al.

b) Applicant argues that **Neckers** et al. teaches the properties of Rose Bengal as a halogenated xanthene but does not teach or suggest the interaction of the agent with ionizing radiation or use of the agent upon interaction with ionizing radiation for treatment of cancer or tumors. Applicant further argues that interaction of the agent with ionizing radiation is affected only by absolute halogen content; the activation of the agent with visible light is fundamentally different from its activation with ionizing radiation; and its conceptual role is to serve as vehicle for concentration and delivery of active halogens.

In response, Neckers, indeed, teach rose bengal, a halogenated or iodinated xanthene, which is principally tetrachlorotetraiodofluorescein, which has an inherent

capacity to rapidly and efficiently concentrate into cellular molecules upon exposure to ionizing radiation; thus, a radiosensitizer or a pharmaceutical agent which in combination with ionizing radiation, finds use in treating diseased tissue. Specifically, Applicant claims a radiosensitizer agent, halogenated xanthene, a known product clearly taught by Neckers and which has the inherent property to concentrate into diseased tissue. Therefore, the product of the present invention is concluded to be inherently anticipated by Neckers.

In response to applicant's argument that Neckers fails to show certain features of applicant's invention, it is noted that the features upon which applicant relies, i.e. "interaction/activation of the agent with/by ionizing radiation", "interaction is affected only by absolute halogen content", are not recited in the rejected claims. Although the claims are interpreted in light of the specification, limitations from the specification are not read into the claims. See *In re Van Geuns*, 988 F.2d 1181, 26 USPQ2d 1057 (Fed. Cir. 1993).

c) Applicant argues that Serafini et al. and Neckers fail to disclose the radiosensitizer as disclosed in the claimed invention, particularly "a radiosensitizer for treatment of cancer and tumors using radiosensitization or ionizing radiation" and Khaw et al. only disclose immunoliposomes for targeted delivery of their contents such as contrast agents to various immunologic targets.

In response to applicant's piecemeal analysis and arguments against Serafini, Neckers, and Khaw individually, one cannot show nonobviousness by attacking the references individually where the rejections are based on combinations of references. See *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In re Merck & Co.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986).

Refer to a) and b) supra for discussion of the relevancy of Serafini and Neckers.

In this case, it would have been obvious to one of ordinary skill in the art at the time of the invention to incorporate the Rose Bengal taught by Serafini or Neckers into the immunoliposomes for target delivery taught by Khaw because Khaw specifically taught that any antineoplastic, contrast, and other pharmacologically active agents can be substituted into the immunoliposomes for use in targeting the elements into desired biologically sensitive specific tissues to allow diagnosis, localization, and therapy and Serafini and Neckers taught application of Rose Bengal into specific tissue as contrast agents. One of ordinary skill in the art at the time of the instant invention would have been motivated to incorporate the teachings of Serafini or Neckers into the immunoliposomes of Khaw because of the multifunctional capacity as achieved in combining treatment and imaging capability in using a single radiosensitization agent for treatment of cancer or tumorous tissues.

18. Accordingly, no claims are allowed.

19. Applicant's amendment necessitated the new grounds of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

20. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Gail Gabel whose telephone number is (703) 305-0807. The examiner can normally be reached on Monday to Thursday from 7:00 AM to 4:30 PM. The examiner can also be reached on alternate Fridays from 7:00 AM to 3:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Long Le, can be reached on (703) 308-3399. The fax phone number for the organization where this application or proceeding is assigned is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-0196.

12/23/01

Gail Gabel
Patent Examiner
Group 1641

LONG V. LE
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

12/26/01

OIPE
JAN 16 2001
PATENT & TRADEMARK OFFICE

LIST OF PUBLICATIONS CITED BY APPLICANT	Attorney Docket No. PHO 107 DIV	Serial No. 09/382,622
	Applicant H. Craig DEES et al	
	Filing Date August 25, 1999	Group 1641

U.S. PATENT DOCUMENTS

*Examiner Initial	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE
	4,599,227	07/08/86	Dees et al	424	38	11/07/83
	4,973,848	11/27/90	Kolobanov et al	250	458.1	07/28/89
	5,128,139	07/07/92	Brown et al	424	450	02/15/91
	5,149,801	09/22/92	Kahl et al	540	145	11/21/90
	5,284,831	02/08/94	Kahl et al	514	21	04/16/92
	5,576,013	11/19/96	Williams et al	424	423	03/21/95
	5,591,422	01/07/97	Hemmi et al	424	362	06/06/95
	5,654,423	08/05/97	Kahl et al	540	145	05/23/95
	5,827,186	10/27/98	Chen et al	600	407	04/11/97
	5,829,448	11/03/98	Fisher et al	128	898	10/30/96
	5,832,931	11/10/98	Wachter et al	128	898	10/30/96
	5,998,597	12/07/99	Fisher et al	536	23.1	12/11/97
	6,042,603	03/28/00	Fisher et al	607	89	05/04/98

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE
	WO 00/25819	05/11/00	General Hospital Corp.			10/29/99
	WO 00/25829	05/11/00	General Hospital Corp.			10/29/99

OTHER PUBLICATIONS
(Including Author, Title, Date, Pertinent Pages)

1) Fisher et al, "Clinical and Preclinical Photodynamic Therapy," *Lasers in Surgery and Medicine*, vol. 17, pp. 2-31, 1995.
2) Young, S. et al, "Gadolinium(III) Texaphyrin: A Tumor Selective Radiation Sensitizer that is Detectable by MRI," Proc. Natl. Acad. Sci., vol. 93, pp. 6610-6615, June, 1996.
3) Bernhard, E.J. et al, "Re-Evaluating Gadolinium(III) Texaphyrin as a Radiosensitizing Agent," *Cancer Research*, vol. 60, pp. 86-91, January 1, 2000.

EXAMINER: Dwayne R. Jones DATE CONSIDERED: 2/23/01

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP form. Draw line through citation if not in conformance and not considered. Include a copy of this form with the next communication to applicant.

LIST OF PUBLICATIONS/MATERIALS CITED BY APPLICANT	Attorney Docket No. PHO-107 DIV	Serial No. 09/382,622
	Applicant Dees et al.	
	Filing Date August 25, 1999	Group 1641

OIPE JC42 JAN 22 2001 PATENT & TRADEMARK OFFICE

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE
SB	4,957,481	9/18/90	Gatenby			

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB-CLASS	FILING DATE

RECEIVED JAN 24 2001 TECH CENTER 1600/2900

OTHER PUBLICATIONS/MATERIALS
(Including Author, Title, Date, Pertinent Pages)

EXAMINER: Dolores R. Dahl	DATE CONSIDERED: 2/20/01

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP form. Draw line through citation if not in conformance and not considered. Include a copy of this form with the next communication to applicant.

Exhibit C

ALL-STATE® LEGAL 800-222-0510 EDS11 RECYCLED

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

2

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
09/382,622	08/25/99	DEES	H PHO-107-DIV

EXAMINER	
GABEL, G	
ART UNIT	PAPER NUMBER
1641	12

HM12/0618

EDWARD D MANZO
COOK ALEX MCFARRON MANZO CUMMINGS
& MEHLER LTD
200 WEST ADAMS STREET SUITE 2850
CHICAGO IL 60606

DATE MAILED: 06/18/01

RECEIVED
JUN 20 2001
COOK, ALEX, McFARRON,
MANZO, CUMMINGS & MEHLER

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Photogen

CASE PHO-107DIV
ATTY. edm/mjm
DUE DATE DKT. BY
CALL U- DKT. BY
RESP. SENT DKT. BY
REVIEWED BY ATTY DATE
OK TO FILE DATE

Interview Summary	Application No.	Applicant(s)	
	09/382,622	DEES ET AL.	
	Examiner	Art Unit	
	Gailene R. Gabel	1641	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Gailene R. Gabel*. (3)_____.

(2) *Mark Murphy*. (4)_____.

Date of Interview: *07 June 2001* .

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☒ No.
If Yes, brief description: .

Claim(s) discussed: *of record* .

Identification of prior art discussed: *of record* .

Agreement with respect to the claims f)☐ was reached. g)☒ was not reached. h)☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *See Continuation Sheet* .

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

i)☐ It is not necessary for applicant to provide a separate record of the substance of the interview(if box is checked).

Unless the paragraph above has been checked, THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Christopher L. Chin

CHRISTOPHER L. CHIN
PRIMARY EXAMINER
GROUP ~~1800~~ 1641

Gailene R. Gabel
6/12/01

Examiner's signature, if required

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)
In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case unless both applicant and examiner agree that the examiner will record same. Where the examiner agrees to record the substance of the interview, or when it is adequately recorded on the Form or in an attachment to the Form, the examiner should check the appropriate box at the bottom of the Form which informs the applicant that the submission of a separate record of the substance of the interview as a supplement to the Form is not required.

It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
 (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: The Office left a voicemail query to Mr. Murphy to verify desired status of the proposed After Final Amendment submitted on 5/29/01. The Office also notified Mr. Murphy that the Amendment, if treated officially, would not have been entered due to new issues and unsupported new recitations incorporated into the claims . As of 6/12/01, the Office has not received telephonic verification to treat the proposed After Final Amendment submission as official; therefore, the paper was neither filed nor entered officially into the application .

Exhibit D

ALL-STATE® LEGAL 800-222-0510 ED511 RECYCLED

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
09/382,622	08/25/99	DEES	PHO-107-DIV

HM12/0725

EDWARD D MANZO
COOK ALEX MCFARRON MANZO CUMMINGS
& MEHLER LTD
200 WEST ADAMS STREET SUITE 2850
CHICAGO IL 60606

EXAMINER	
GABEL, G	
ART UNIT	**PAPER NUMBER**
1641	15

DATE MAILED: 07/25/01

RECEIVED
JUL 30 2001
COOK, ALEX, McFARRON,
MANZO, CUMMINGS & MEHLER

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Photogen

CASE	PHO-107 DIV
ATTY.	asm/mjm
DUE DATE	DKT. BY
CALL UP	DKT. BY
RESP. SENT	DKT. BY
REVIEWED BY ATTY	DATE
OK TO FILE	DATE

Advisory Action	Application No. 09/382,622	Applicant(s) DEES ET AL.	
	Examiner Gailene R. Gabel	Art Unit 1641	

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 29 June 2001 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE. Therefore, further action by the applicant is required to avoid abandonment of this application. A proper reply to a final rejection under 37 CFR 1.113 may only be either: (1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114.

PERIOD FOR REPLY [check either a) or b)]

a) ☒ The period for reply expires 4 months from the mailing date of the final rejection.

b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action, or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.
ONLY CHECK THIS BOX WHEN THE FIRST REPLY WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

1. ☐ A Notice of Appeal was filed on _____. Appellant's Brief must be filed within the period set forth in 37 CFR 1.192(a), or any extension thereof (37 CFR 1.191(d)), to avoid dismissal of the appeal.

2. ☒ The proposed amendment(s) will not be entered because:

 (a) ☒ they raise new issues that would require further consideration and/or search (see NOTE below);

 (b) ☒ they raise the issue of new matter (see Note below);

 (c) ☒ they are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or

 (d) ☐ they present additional claims without canceling a corresponding number of finally rejected claims.

 NOTE: *See Continuation Sheet*.

3. ☐ Applicant's reply has overcome the following rejection(s): _____.

4. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).

5. ☒ The a)☐ affidavit, b)☐ exhibit, or c)☒ request for reconsideration has been considered but does NOT place the application in condition for allowance because: *See Continuation Sheet*.

6. ☐ The affidavit or exhibit will NOT be considered because it is not directed SOLELY to issues which were newly raised by the Examiner in the final rejection.

7. ☐ For purposes of Appeal, the proposed amendment(s) a)☐ will not be entered or b)☐ will be entered and an explanation of how the new or amended claims would be rejected is provided below or appended.

 The status of the claim(s) is (or will be) as follows:

 Claim(s) allowed: *NONE*.

 Claim(s) objected to: *NONE*.

 Claim(s) rejected: *1-10,12-16,18-20,23,25-27,50-52 and 54-59*.

 Claim(s) withdrawn from consideration: _____.

8. ☐ The proposed drawing correction filed on _____ is a)☐ approved or b)☐ disapproved by the Examiner.

9. ☐ Note the attached Information Disclosure Statement(s)(PTO-1449) Paper No(s). _____.

10. ☐ Other:

LONG V. LE
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

07/24/01

1/23/01

Continuation of 2. NOTE: Applicant's amendment of claim 1 introduced new 35 USC 112 first and second paragraph issues requiring further consideration and search in reciting "halogenated xanthene interacting with ionizing radiation ... to enhance therapeutic efficacy of ionizing radiation". For example, it is unclear as to whether the term "interacting" as recited would encompass "activating", "inactivating"; thereby, reciting a broader scope enabled by the claimed agent. Further, such recitation does not appear to be fully and literally supported by the specification; thereby, introducing new matter issues into the claimed invention .

Continuation of 5. does NOT place the application in condition for allowance because: new matter and new consideration issues have been introduced into claim 1. Further, the claims, as currently recited, fail to obviate the prior art of record.

$3t, 4y, t3$

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COOK, ALEX, McFARRON,
MANZO, CUMMINGS & MEHLER

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
09/382,622	08/25/99	DEES	PHO-107-DIV

EXAMINER	
GABEL G	
ART UNIT	**PAPER NUMBER**
1641	19

DATE MAILED: 09/13/01

4M22/0913

EDWARD D MANZO
COOK ALEX MCFARRON MANZO CUMMINGS
& MEHLER LTD
200 WEST ADAMS STREET SUITE 2850
CHICAGO IL 60606

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Photogen

CASE	PHO-107 DIV
ATTY.	eam/mym
DUE DATE	DKT. BY
CALL UP	DKT. BY
RESP. SENT	DKT. BY
REVIEWED BY ATTY	DATE
OK TO FILE	DATE

3 mo. Response to O.A. due 12/13/01

Office Action Summary	Application No.	Applicant(s)	
	09/382,622	DEES ET AL.	
	Examiner	Art Unit	
	Gailene R. Gabel	1641	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *08 July 2001* .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-10,14,15,18-20,50-52 and 55-57* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-10,14,15,18-20,50-52 and 55-57* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: .

DETAILED ACTION

Continued Examination Under 37 CFR 1.114

1. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 7/8/01 has been entered.

Amendment Entry

2. Applicant's amendment and response filed 7/8/01 in Paper No. 14 is acknowledged and has been entered. Claims 12-13, 16, 23, 25-27, 54, 58, and 59 have cancelled. Claims 1, 4-7, 9-10, 14-15, 18, 51-52, and 55 have been amended. Accordingly, claims 1-10, 14-15, 18-20, 50-52, and 55-57 are pending and under examination.

Rejections Withdrawn

Claim Rejections - 35 USC § 112/103

3. The rejection of claims 15-16, 23, 25-27, and 54 under 35 U.S.C. 112, second paragraph, is now moot in light of Applicant's cancellation of the claims or otherwise withdrawn in light of Applicant's amendment.

4. The rejection of claims 25-27 under 35 U.S.C. 103(a) as being unpatentable over Serafini et al. (Journal of Nuclear Medicine, 1975) or Neckers D. (Journal of Photochemistry and Photobiology, A: Chemistry 47: 1-29 (1989)) in view of Khaw et al. (US 5,780,052) is now moot in light of Applicant's cancellation of the claims.

Rejections Maintained

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless -
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

5. Claims 1-3, 5-8, and 12-13 stand rejected under 35 U.S.C. 102(b) as being inherently anticipated by Serafini et al. (Journal of Nuclear Medicine, 1975) for reasons of record.

6. Claims 1-3, 5-9, and 12-13 stand rejected under 35 U.S.C. 102(b) as being inherently anticipated by Neckers D. (Journal of Photochemistry and Photobiology, A: Chemistry ~~47~~: 1-29 (1989)) for reasons of record.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and

> the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

7. Claims 4, 14, and 18-20 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Serafini et al. (Journal of Nuclear Medicine, 1975) or Neckers D. (Journal of Photochemistry and Photobiology, A: Chemistry 47: 1-29 (1989)) in view of Khaw et al. (US 5,780,052) for reasons of record.

8. Claim 10, 51, 52, and 56-57 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Serafini et al. (Journal of Nuclear Medicine, 1975) or Neckers D. (Journal of Photochemistry and Photobiology, A: Chemistry 47: 1-29 (1989)) in view of Norman et al. (Invest Radiol, 26: S120-S121, 1991) for reason of record.

Response to Arguments

9. Applicant's arguments filed 7/8/01 have been fully considered but they are not persuasive.

A) Applicant argues that the agents in the present invention are "medicinal drugs" that can be administered to patients, i.e. medicinal purposes, to improve efficacy of radiation therapy. Specifically, Applicant contends that the existing reagent grade commercial product, i.e. reagent grade Rose Bengal, used in industrial laboratory is completely distinct from the claimed drug product for use in radiation therapy, specifically used as a radiosensitizer for treatment of cancers and tumors comprising halogenated xanthene which interacts with ionizing radiation applied to said cancer or tumor.

In response, the discovery of a new property of a known product, in this case, Rose Bengal as a radiosensitizer, does not render the product novel; unless otherwise, rendered novel or nonobvious from modification or variation therefrom, i.e. "ionized Rose Bengal" or "non-radioactive Rose Bengal" or "pharmaceutical Rose Bengal" that is structurally different, novel, and nonobvious, i.e. with different pH (as an example) from all other commercially known Rose Bengal.

B) Applicant argues that the claims are directed to novel and nonobvious combinations of 1) radiosensitizer agent comprising a halogenated xanthene, 2) interacting with ionizing radiation applied to a cancer or tumor, 3) to enhance therapeutic efficiency of the ionizing radiation. Applicant argues that none of the cited references disclose or suggest that a halogenated xanthene or Rose Bengal is a radiosensitizer or radiosensitizer agent for use with ionizing radiation. Specifically, Serafini et al. disclose Rose Bengal and a use thereof but fails to disclose Applicant's

specific "radiosensitizer combination" aforementioned. Therefore, Rose Bengal sold for "use" for diagnostic purposes in Serafini et al. would have a different label as a different drug, i.e. commercial entity. Applicant also contends that Serafini et al. does not teach or suggest the agent of the claimed invention as a radiosensitizer agent for use with radiosensitization or ionizing radiation in the treatment of cancer or tumors. Specifically, Applicant argues that Serafini et al.'s use of Rose Bengal is completely different from the radiosensitizer agent of claim 1.

In response, Examiner concurs that Serafini et al. does not disclose **use** of Rose Bengal as a radiosensitizer agent for use with ionizing radiation to treat cancer or tumor. Examiner concurs that Serafini et al.'s **use** is completely different from **use** as the radiosensitizer agent of claim 1. Applicant, however, does not appear to claim the **use** of Rose Bengal as radiosensitizer agent but appears to claim Rose Bengal as the radiosensitizer agent, or otherwise, Rose Bengal as the product itself, in combined use with ionizing radiation; thus a radiosensitizer agent. Serafini et al. teach a different **use** of Rose Bengal but, nevertheless, use the same Rose Bengal having the same structure and functional properties as the radiosensitizer agent recited in claim 1. Therefore, absent any evidentiary showing that the structure of the Rose Bengal taught by Serafini et al. distinctly differs from the claimed invention, absent evidentiary showing that the Rose Bengal as taught by Serafini et al. would not have exhibited the same (inherent) property as a radiosensitizing agent upon exposure to ionizing radiation, it is maintained that the Rose Bengal taught by Serafini et al. is the same compound as the radiosensitizer agent taught in the claimed invention. As an example, an "ionized Rose

Bengal" or "non-radioactive Rose Bengal" that is structurally different, novel, and nonobvious, i.e. with different pH (as an example) from all other commercially known Rose Bengal.

C) Applicant argues that Neckers does not teach or suggest the agent of the claimed invention as a radiosensitizer agent for use with radiosensitization or ionizing radiation in the treatment of cancer or tumors. Applicant argues that Necker's teaching of use in optical radiation of Rose Bengal is completely different from the use with ionizing radiation as radiosensitizer agent of claim 1.

In response, Examiner concurs that Neckers does not disclose **use** of Rose Bengal as a radiosensitizer agent for use with ionizing radiation to treat cancer or tumor. Examiner concurs that Necker's **use** is completely different from use as the radiosensitizer agent of claim 1. Applicant, however, does not appear to claim the **use** of Rose Bengal as radiosensitizer agent but appears to claim Rose Bengal as the radiosensitizer agent, or otherwise, Rose Bengal as the product itself, in combined use with ionizing radiation; thus a radiosensitizer agent. Neckers teaches a different **use** of Rose Bengal but, nevertheless, teaches the same Rose Bengal having the same structure and functional properties as the radiosensitizer agent recited in claim 1. Therefore, absent any evidentiary showing that the structure of the Rose Bengal taught by Neckers distinctly differs from the claimed invention, absent evidentiary showing that the Rose Bengal as taught by Neckers would not have exhibited the same (inherent) property as a radiosensitizing agent upon exposure to ionizing radiation, it is maintained

that the Rose Bengal taught by Serafini et al. is the same compound as the radiosensitizer agent taught in the claimed invention. As an example, an "ionized Rose Bengal" or "non-radioactive Rose Bengal" that is structurally different, novel, and nonobvious, i.e. with different pH (as an example) from all other commercially known Rose Bengal.

D) Applicant argues that the combination of Serafini et al. or Neckers with Khaw et al. does not teach or suggest the agent of the claimed invention as a radiosensitizer agent for use with radiosensitization or ionizing radiation in the treatment of cancer or tumors. Applicant argues that Khaw et al. does not correct the deficiencies of Serafini et al. and Neckers. Specifically Khaw et al. does not suggest **use** of halogenated xanthene as radiosensitizer agent such as recited in claim 1.

In response to applicant's arguments against the references individually, one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. See *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In re Merck & Co.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986). In this case, Examiner concurs that Serafini et al. or Neckers combined with Khaw et al. do not suggest **use** of Rose Bengal as a radiosensitizer agent for use with ionizing radiation to treat cancer or tumor. Examiner concurs that **use** of the combined references is completely different from the **use** of the radiosensitizer agent of the rejected claims. However, the combined references, nevertheless, suggest the same Rose Bengal having the same structure and functional properties as the radiosensitizer

agent recited in the rejected claims. Therefore, absent any evidentiary showing that the structure of the Rose Bengal suggested by the combined references distinctly differs from the claimed invention, absent evidentiary showing that the Rose Bengal as suggested by the same references would not have exhibited the same (inherent) property as a radiosensitizing agent upon exposure to ionizing radiation, it is maintained that the Rose Bengal suggested by the combined references is the same compound as the radiosensitizer agent taught in the claimed invention. As an example, an "ionized Rose Bengal" or "non-radioactive Rose Bengal" that is structurally different, novel, and nonobvious, i.e. with different pH (as an example) from all other commercially known Rose Bengal.

E) Applicant argues that the combination of Serafini et al. or Neckers with Norman does not teach or suggest the agent of the claimed invention as a radiosensitizer agent for use with radiosensitization or ionizing radiation in the treatment of cancer or tumors. Applicant argues that Norman does not correct the deficiencies of Serafini et al. and Neckers. Specifically Norman does not suggest **use** of halogenated xanthene as radiosensitizer agent such as recited in claim 1.

In response to applicant's arguments against the references individually, one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. See *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In re Merck & Co.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986). In this case, Examiner concurs that Serafini et al. or Neckers combined with Norman do

not disclose **use** of Rose Bengal as a radiosensitizer agent for use with ionizing radiation to treat cancer or tumor. Examiner concurs that **use** of the combined references is completely different from the **use** of the radiosensitizer agent of the rejected claims. However, the combined references, nevertheless, suggests the same Rose Bengal having the same structure and functional properties as the radiosensitizer agent recited in the rejected claims. Therefore, absent any evidentiary showing that the structure of the Rose Bengal suggested by the combined references distinctly differs from the claimed invention, absent evidentiary showing that the Rose Bengal as suggested by the same references would not have exhibited the same (inherent) property as a radiosensitizing agent upon exposure to ionizing radiation, it is maintained that the Rose Bengal suggested by the combined references is the same compound as the radiosensitizer agent taught in the claimed invention. As an example, an "ionized Rose Bengal" or "non-radioactive Rose Bengal" that is structurally different, novel, and nonobvious, i.e. with different pH (as an example) from all other commercially known Rose Bengal.

10. No claims are allowed.

11. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Gail Gabel whose telephone number is (703) 305-0807. The examiner can normally be reached on Monday to Thursday from 7:00 AM to 4:30 PM. The examiner can also be reached on alternate Fridays from 7:00 AM to 3:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Long Le, can be reached on (703) 308-3399. The fax phone number for the organization where this application or proceeding is assigned is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-0196.

9/10/01

Gail Gabel
Patent Examiner
Group 1641

LONG V. LE
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

09/10/01

Attachment for PTO-948 (Rev. 03/01, or earlier)
6/18/01

The below text replaces the pre-printed text under the heading, "Information on How to Effect Drawing Changes," on the back of the PTO-948 (Rev. 03/01, or earlier) form.

INFORMATION ON HOW TO EFFECT DRAWING CHANGES

1. Correction of Informalities -- 37 CFR 1.85

New corrected drawings must be filed with the changes incorporated therein. Identifying indicia, if provided, should include the title of the invention, inventor's name, and application number, or docket number (if any) if an application number has not been assigned to the application. If this information is provided, it must be placed on the front of each sheet and centered within the top margin. If corrected drawings are required in a Notice of Allowability (PTOL-37), the new drawings **MUST** be filed within the **THREE MONTH** shortened statutory period set for reply in the Notice of Allowability. Extensions of time may NOT be obtained under the provisions of 37 CFR 1.136(a) or (b) for filing the corrected drawings after the mailing of a Notice of Allowability. The drawings should be filed as a separate paper with a transmittal letter addressed to the Official Draftsperson.

2. Corrections other than Informalities Noted by Draftsperson on form PTO-948.

All changes to the drawings, other than informalities noted by the Draftsperson, **MUST** be made in the same manner as above except that, normally, a highlighted (preferably red ink) sketch of the changes to be incorporated into the new drawings **MUST** be approved by the examiner before the application will be allowed. No changes will be permitted to be made, other than correction of informalities, unless the examiner has approved the proposed changes.

Timing of Corrections

Applicant is required to submit the drawing corrections within the time period set in the attached Office communication. See 37 CFR 1.85(a).

Failure to take corrective action within the set period will result in **ABANDONMENT** of the application.

Exhibit F

Attorney Docket No: PHO-107 DIV

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Dees et al.

Serial No.: 09/382,622

Filed: August 25, 1999

For: High Energy Phototherapeutics Agents

Examiner: G. Gabel

Art Unit: 1641

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Date of Deposit March 12, 2002

I hereby certify that this correspondence is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to: Commissioner for Patents, Washington, D.C. 20231

Name Rachelle Hammarquist
(typed or printed)

Signature

March 12, 2002

Commissioner for Patents
Washington D.C. 20231

AMENDMENT C

Please amend the above-identified application as follows:

IN THE CLAIMS:

Please add the following new claims:

60 (New). The radiosensitizer agent of Claim 10, wherein said halogenated xanthene comprises Rose Bengal.

61 (New). The radiosensitizer agent of Claim 10 wherein said halogenated xanthene includes as a functional derivative at least one targeting moiety selected from the group consisting of

deoxyribonucleic acid (DNA), ribonucleic acid (RNA), amino acids, proteins, antibodies, ligands, haptens, carbohydrate receptors or complexing agents, lipid receptors or complexing agents, protein receptors or complexing agents, chelators, encapsulating vehicles short- or long-chain aliphatic or aromatic hydrocarbons, aldehydes, ketones, alcohols, esters, amides, amines, nitriles, and azides.

62 (New). The radiosensitizer agent of Claim 10 wherein said halogenated xanthene also is an imaging contrast agent.

63 (New). The radiosensitizer agent of Claim 10 wherein said halogenated xanthene is encapsulated in a delivery vehicle.

64 (New). The radiosensitizer agent of Claim 51, wherein said halogenated xanthene comprises Rose Bengal.

65 (New). The radiosensitizer agent of Claim 51 wherein said halogenated xanthene includes as a functional derivative at least one targeting moiety selected from the group consisting of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), amino acids, proteins, antibodies, ligands, haptens, carbohydrate receptors or complexing agents, lipid receptors or complexing agents, protein receptors or complexing agents, chelators, encapsulating vehicles short- or long-chain aliphatic or aromatic hydrocarbons, aldehydes, ketones, alcohols, esters, amides, amines, nitriles, and azides.

66 (New). The radiosensitizer agent of Claim 51 wherein said halogenated xanthene also is an imaging contrast agent.

67 (New). The radiosensitizer agent of Claim 51 wherein said halogenated xanthene is encapsulated in a delivery vehicle.

REMARKS

Request For Entry of Amendment

Applicants are filing herewith a Notice of Appeal of the Office Action of September 13. 2001 in the above-identified application. In order to put the application in a better condition for appeal, Applicants are adding new claims dependent on independent Claims 10 and 51. These are similar to dependent Claims 2, 4, 5 and 18 which depend on independent Claim 1. It is believed that no new matter is being added and no further search is needed for these claims.

As Applicants have previously paid for 32 claims and only 20 are now pending, it is not believed that any further fee is needed for these 8 new claims. If any further fee is needed for this amendment or appeal, please charge our deposit account 50/1039.

Favorable consideration is earnestly solicited.

Respectfully submitted,

Date: March 12, 2002

Mark J. Murphy
Registration No. 34,225

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Exhibit G

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LIFE SCIENCE DICTIONARY

Search Results

Search Terms: AND **Search Definitions:**

Contains this Begins with this

Total searching time: 0.00sec

Searching Category	User input query
DEFINITION search string	radiosensitizer
Number of Results	1

1. **radiosensitizer**
 Author: Guo Li
 Definition:
 A radiosensitizer is a substance given to a patient to make the intended target (such as a tumor) more susceptible to the effects of radiotherapy.

END

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Exhibit H

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A CLINICAL GUIDE TO CANCER NURSING

A Companion to
Cancer Nursing, Third Edition

Susan L. Groenwald • Margaret Hansen Frogge
Michelle Goodman • Connie Henke Yarbro

At last there is a quick and helpful reference for any nurse caring for patients with cancer in a clinical setting! *A Clinical Guide to Cancer Nursing* derives its information from and follows the general outline of the best-selling *Cancer Nursing: Principles and Practice, Third Edition*-so that either book may be used as a companion to the other.

FEATURES

- Contributions from 65 nationally recognized experts
- Comprehensive, yet portable guide for all nurses in clinical practice



JONES AND BARTLETT PUBLISHERS

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A Companion to *Cancer Nursing, Third Edition*

Groenwald
Frogge
Goodman
Yarbro



Jones
and
Bartlett

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The selection and dosage of drugs presented in this book are in accord with standards accepted at the time of publication. The editors and the publisher have made every effort to provide accurate information. However, research, clinical practice, and government regulations often change the accepted standard in this field. Before administering any drug, the reader is advised to check the manufacturer's product information sheet for the most up-to-date recommendations on dosage, precautions, and contraindications. This is especially important in the case of drugs that are new or seldom used.

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10. Chemotherapy 84
11. Biotherapy 119
12. Bone Marrow Transplantation 133

- *Indirect hit occurs when ionization takes place in medium (mostly water) surrounding molecular structures in cell*
 - Results in free radical that may trigger variety of chemical reactions producing compounds toxic to cell
 - More likely to occur than direct hit
- *Radiosensitivity maximum just before and during actual cell division*
- *Sensitivity of cells to irradiation is directly proportional to their reproductive activity (radiation most effective during mitosis) and inversely proportional to their degree of differentiation (the more differentiated, the less sensitive to radiation)*
- *Cell death from radiation can be immediate or delayed*
- *Radiation can inhibit cell from reproducing*
- *Other factors that directly affect biologic response to radiation include*
 - *Oxygen effect:* Well-oxygenated tumors have greater response to radiation
 - *Linear energy transfer (LET):* Rate at which energy is lost from different types of radiations while traveling through matter
 - Higher LET has greater probability of interacting with matter and producing more direct hits
 - *Relative biologic effectiveness:* Compares dose of test radiation with dose of standard radiation that produces same biologic response

 - *Fractionation:* Division of total dose of radiation into a number of equal fractions to accomplish
 - Repair
 Of intracellular damage by normal cells between daily dose fractions
 Major advantage of fractioning dosages
 - Redistribution (within cell cycle)
 With subsequent daily doses of radiation, more and more tumor cells are delayed in cell cycle and reach mitosis as the next dose is given, increasing cell kill
 - Repopulation (new growth of normal tissue)
 Takes place some time during fractionated treatment course
 Fractionation favors normal tissue while still eradicating tumor
 - Reoxygenation
 Oxygenated cells are more radiosensitive
 Fractionating dose allows time between treatments for tumors to reoxygenate

Chemical and thermal modifiers of radiation

1. Radiosensitizers

- *Increase radiosensitivity of tumor cells*
- *Achieve greater cell kill*
- *Prevent repair of cellular radiation damage*
- *Side effects include*
 - Neurotoxicity
 - CNS symptoms of somnolence, confusion, and transient coma
 - Dose-related nausea and vomiting

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- *Results are disappointing because of severe toxicity and lack of significant efficacy*

2. Radioprotectors

- *Protect aerated (nontumor) cells while having limited effect on hypoxic (tumor) cells*
- *Increase therapeutic ratio by promoting repair of irradiated normal tissues*
- *Nursing responsibilities include*
 - Providing patient and family education
 - Participating in obtaining informed consent
 - Administering investigational agents
 - Timing and coordinating drug administration with radiotherapy treatment
 - Observing and documenting expected and previously unreported effects and side effects
 - Managing side effects, including developing interventions for those newly observed

3. Hyperthermia

- *Heat is cytotoxic to cancer cells*
- *Hyperthermia combined with radiotherapy produces greater effect than either modality alone*
- *Parameters that may influence tumor response to combined hyperthermia and radiation therapy include*
 - Tumor size
 - Histologic findings
 - Disease site
 - Total dose of radiation and dose per fraction

 - Thermal dose
 - Total and weekly number of hyperthermia sessions
 - Sequencing of hyperthermia and radiation
- *Hyperthermia is achieved by*
 - Immersion of local area in heated bath
 - Ultrasound
 - Microwaves
 - Interstitial implants
 - Perfusion techniques
- *Side effects include*
 - Local skin reaction
 - Pain
 - Fever
 - Gastrointestinal effects
 - Cardiac arrhythmias
 - Late effects such as necrosis and ulceration; these are generally not severe enough to discontinue use
- *Nursing responsibilities*
 - Pretreatment evaluation phase
 - Assess for
 Suitability for treatment
 Ability to tolerate treatment
 Cardiac and neurologic status
 Presence of metal objects
 - Provide thorough patient and family education
 - Treatment phase
 - Monitor vital signs
 - Prepare patient's gastrointestinal tract

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Exhibit 1

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CANCER NURSING

Principles and Practice

FOURTH EDITION

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The selection and dosage of drugs presented in this book are in accord with standards accepted at the time of publication. The authors, editors, and publisher have made every effort to provide accurate information. However, research, clinical practice, and government regulations often change the accepted standard in this field. Before administering any drug, the reader is advised to check the manufacturer's product information sheet for the most up-to-date recommendations on dosage, precautions, and contraindications. This is especially important in the case of drugs that are new or seldom used.

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Thus fractionation favors normal tissue while still eradicating tumor.

Reoxygenation Reoxygenation is the fourth consideration favoring fractionation of the radiation dose. Whereas normal tissues usually are well oxygenated, tumors characteristically range from normal to hypoxic to anoxic. As discussed earlier, radiosensitivity is closely related to oxygen tension in the tumor cell; hypoxic or anoxic cells generally are radioresistant, whereas oxygenated cells are radiosensitive. Fractionating the dose allows time between treatments for the tumor to reoxygenate.

Tissue and organ response to radiation is based on the sensitivity of cellular components. It is important to note that tissues and organs are composed of more than one cell category, each cell category having different degrees of radiosensitivity. A second factor in determining tissue response is related to the parenchymal versus stromal substance found in that tissue. The parenchyma is composed of cells characteristic of the tissue or organ, and if those cells are radiosensitive (e.g., the testis), ionizing radiation has its greatest impact on the parenchyma. However, if parenchymal tissue is relatively radioresistant (e.g., the spinal cord), radiation response in that organ is due to the indirect effects on the stromal components (especially the vasculature) that support the parenchyma. Table 13-2 lists various organs according to their degree of radiosensitivity as measured by parenchymal hypoplasia.

TABLE 13-2 Degree of Radiosensitivity of Various Organs Based on Parenchymal Hypoplasia

Organ	Radiosensitivity
Lymphoid organs, bone marrow, blood, testes, ovaries, intestines	High
Skin, cornea, oral cavity, esophagus, rectum, bladder, vagina, cervix, ureters	Fairly high
Optic lens, stomach, growing cartilage, fine vasculature, growing bone	Medium
Mature cartilage or bone, salivary glands, respiratory organs, kidneys, liver, pancreas, thyroid, adrenals, pituitary gland	Fairly low
Muscle, brain, spinal cord	Low

CHEMICAL AND THERMAL MODIFIERS OF RADIATION

Radiosensitizers and Radioprotectors

The goal of radiotherapy is to achieve maximum tumor cell kill while minimizing injury to normal tissues (therapeutic ratio). Efforts to improve the therapeutic ratio have resulted in the development of certain compounds that act to increase the radiosensitivity of tumor cells or to protect normal cells from radiation effect. Combined modality therapy with both radiation and certain cytotoxic agents also takes advantage of enhanced tumor cell kill. Drugs such as doxorubicin, actinomycin-D, cyclophosphamide, bleomycin, and cisplatin often are used along with radiation to achieve greater cell kill than either therapy could achieve if used independently. When used alone, chemical modifiers of radiation therapy (radiosensitizers), however, are not generally cytotoxic like the chemotherapeutic agents.

Phillips[16] proposed several definitions useful in describing the various interactions of radiation with other agents. *Enhancement* or *potentiation* describes any radiation effect that is greater in the presence of the chemical than in its absence. If the effect is less than that caused by the most active agent in the combination, then this is known as *interference*. *Antagonism* is the term used to describe an outcome less than that of the least effective agent in a given combination. In clinical radiotherapy, enhancement by noncytotoxic sensitizers is called *radiosensitization*. Antagonism by protective compounds is called *radioprotection*.

Radiosensitizers are compounds that apparently promote fixation of the free radicals produced by radiation damage at the molecular level. The mechanism of this action is similar to the oxygen effect described earlier, in which biochemical reactions in the damaged molecules prevent repair of the cellular radiation damage. Free radicals (such as $OH^{\bullet}$) are captured by the electron-affinic radiosensitizers, rendering the molecules incapable of repair.

The two most biologically active radiosensitizing compounds first tested in phase II and III studies were metronidazole (Flagyl) and misonidazole (RO-07-0582). Major side effects are neurotoxicity, including peripheral neuropathies; somnolence, confusion, and transient coma. Nausea and vomiting are also frequent side effects that seem to be dose-related.

Early clinical trials using misonidazole as a radiosensitizer indicated some degree of effectiveness in treatment of squamous carcinoma of the head and neck and of the uterine cervix. Overall results were disappointing, however, due to severe toxicity and only marginal improvement in tumor control.[17] Misonidazole is the only such substance to have undergone extensive clinical trial evaluation. Misonidazole has been shown to increase the cytotoxicity of alkylating agents, nitrosoureas, 5-fluorouracil (5-FU), cyclophosphamide, and melphalan. However, the side effects commonly experienced with these agents also are apparently enhanced by the addition of misonidazole. This nonselective enhancement significantly detracts from the potential benefits to be gained.

The compound SR-2508 (etanidazole) has been tested with encouraging results in phase II and III trials.[18] This member of the nitroimidazole group of compounds appears to be less toxic to the CNS tissue than misonidazole and has also been shown to cross the blood-brain barrier in limited quantity.

Early studies[19] with nitroimidazoles used in vitro showed that these radiosensitizers are also capable of cytotoxic activity in hypoxic cells after periods of long exposure. The high doses required to achieve actual cell kill in vitro have, however, prohibited their use in vivo for this purpose.

The established basis for use of chemical radiosensitizers is based on extensive laboratory research.[20] However, clinical trials thus far have failed to establish the overall efficacy of radiosensitizers as adjuncts to radiation therapy in the clinical setting.

Radioprotectors are compounds that can protect oxygenated (nontumor) cells while having a limited effect on hypoxic (tumor) cells. This selective action serves to increase the therapeutic ratio by promoting the repair of irradiated normal tissues. Repair or return to a nondamaged state takes place through the chemical process of reduction. Free electrons are captured by the radioprotective substance and thus are unavailable to participate in further chemical reactions that lead to cellular damage. This process can be viewed as the opposite of what occurs when radiosensitizers are used.

The sulfhydryl groups contained in the nonprotein fraction of most cells aid in the reduction process following radiation damage. Thiophosphate compounds (such as cysteine and cysteamine) containing sulfhydryl and aminopropyl groups were among the earliest radioprotectors synthesized. The compound that appears to be most useful at present is designated WR-2721.[21]

The study of radiosensitizers and radioprotectors has extended to phase II and III clinical trials in the continued effort to achieve better results in cancer treatment with radiotherapy. With combined therapy, toxicity can be increased and patient comfort may be compromised. Some nursing responsibilities in the use of radiosensitizers and radioprotectors would include:

1. providing patient and family education
2. participating in obtaining informed consent
3. administering investigational agents
4. timing and coordinating drug administration with radiotherapy treatment
5. observing and documenting expected and previously unreported effects and side effects
6. managing side effects, including developing interventions for those newly observed

A thorough discussion of the nursing implications for patients receiving chemical modifiers of radiation can be found in Noll.[22]

Combined Modality Therapy

Treatment of cancer with any single modality (surgery, chemotherapy, or radiation) does not always produce the desired effect of tumor eradication. Chemotherapy and radiation therapy produce dose-limiting side effects that govern the extent of single modality treatment. In order to increase or improve the therapeutic index (ratio of tumor control versus normal tissue damage), various combinations of chemotherapy and radiation have been studied.

Ideally, a chemotherapeutic agent (or combination of agents) will shrink a tumor when given *prior* to local radiation (neoadjuvant chemotherapy); enhance or increase radiation cell kill when given *during* radiation (concomitant therapy); or control micrometastases and subclinical disease *after* a course of radiation (adjuvant therapy). Radiation and chemotherapy sometimes are given on a planned, alternating schedule using the so-called *sandwich technique.* This approach utilizes a split course of radiation in which the patient is treated with chemotherapy during a planned break in the total course of radiation therapy.[23]

Combined modality therapy is being used in the treatment of a variety of cancer types, including squamous cell cancer of the cervix, anus, head and neck, and lung. Cancers of the bladder, esophagus, pancreas, and stomach frequently are treated with both modalities in varying schedules. Vigorous combined modality therapy has allowed organ preservation for some individuals with carcinoma of the larynx, bladder, or anus.[23,24]

Some of the chemotherapeutic agents in common use for their radiosensitizing effect include cisplatin, methotrexate, doxorubicin, vinblastin, VP-16, mitomycin C, 5-fluorouracil, actinomycin D, and bleomycin. As would be expected, combined-modality therapy has the potential for enhanced side effects as well as enhanced tumor effect. Organ systems at greatest risk for toxicity are the gastrointestinal, integumentary, and myeloproliferative systems.

Nursing implications for care of the patient receiving combined-modality therapy are based on the individual's treatment plan and an understanding of the principles of combined therapies. Enhanced side effects of both radiation and chemotherapy are common. Awareness of the drugs and radiation site and dose are essential elements in anticipating toxicities, monitoring acuity, and implementing appropriate care. Hirshfield-Bartek[25] and Held and Volpe[23] developed detailed nursing guidelines for assessment and management of the patient receiving combined-modality therapy.

Hyperthermia

The use of hyperthermia to achieve a synergistic effect with radiotherapy has been studied and applied in clinical situations with considerable enthusiasm. Although it is technically arguable whether hyperthermia actually sensitizes tumor cells to radiation effect or simply combines to produce a greater effect than either modality can achieve on its own, it is generally agreed that this combined technique is warranted and research continues.[26–29]

The biological basis for combining hyperthermia with radiation involves several factors. Heat is cytotoxic to can-

Exhibit J

Subpart F—Labeling Claims for Drugs in Drug Efficacy Study

201.200 Disclosure of drug efficacy study evaluations in labeling and advertising.

Subpart G—Specific Labeling Requirements for Specific Drug Products

201.300 Notice to manufacturers, packers, and distributors of glandular preparations.
201.301 Notice to manufacturers, packers, and distributors of estrogenic hormone preparations.
201.302 Notice to manufacturers, packers, and distributors of drugs for internal use which contain mineral oil.
201.303 Labeling of drug preparations containing significant proportions of wintergreen oil.
201.304 Tannic acid and barium enema preparations.
201.305 Isoproterenol inhalation preparations (pressurized aerosols, nebulizers, powders) for human use; warnings.
201.306 Potassium salt preparations intended for oral ingestion by man.
201.307 Sodium phosphates; package size limitation, warnings, and directions for over-the-counter sale.
201.308 Ipecac syrup; warnings and directions for use for over-the-counter sale.
201.309 Acetophenetidin (phenacetin)-containing preparations; necessary warning statement.
201.310 Phenindione; labeling of drug preparations intended for use by man.
201.311 [Reserved]
201.312 Magnesium sulfate heptahydrate; label declaration on drug products.
201.313 Estradiol labeling.
201.314 Labeling of drug preparations containing salicylates.
201.315 Over-the-counter drugs for minor sore throats; suggested warning.
201.316 Drugs with thyroid hormone activity for human use; required warning.
201.317 Digitalis and related cardiotonic drugs for human use in oral dosage forms; required warning.
201.319 Water-soluble gums, hydrophilic gums, and hydrophilic mucilloids (including, but not limited to agar, alginic acid, calcium polycarbophil, carboxymethylcellulose sodium, carrageenan, chondrus, glucomannan ((B-1,4 linked) polymannose acetate), guar gum, karaya gum, kelp, methylcellulose, plantago seed (psyllium), polycarbophil tragacanth, and xanthan gum) as active ingredients; required warnings and directions.
201.320 Warning statements for drug products containing or manufactured with chlorofluorocarbons or other ozone-depleting substances.
201.322 Over-the-counter drug products containing internal analgesic/antipyretic active ingredients; required alcohol warning.
201.323 Aluminum in large and small volume parenterals used in total parenteral nutrition.
APPENDIX A TO PART 201—EXAMPLES OF GRAPHIC ENHANCEMENTS USED BY FDA

AUTHORITY: 21 U.S.C. 321, 331, 351, 352, 353, 355, 358, 360, 360b, 360gg-360ss, 371, 374, 379e; 42 U.S.C. 216, 241, 262, 264.

SOURCE: 40 FR 13998, Mar. 27, 1975, unless otherwise noted.

Subpart A—General Labeling Provisions

§ 201.1 Drugs; name and place of business of manufacturer, packer, or distributor.

(a) A drug or drug product (as defined in § 320.1 of this chapter) in finished package form is misbranded under section 502 (a) and (b)(1) of the act if its label does not bear conspicuously the name and place of business of the manufacturer, packer, or distributor. This paragraph does not apply to any drug or drug product dispensed in accordance with section 503(b)(1) of the act.

(b) As used in this section, and for purposes of section 502 (a) and (b)(1) of the act, the manufacturer of a drug product is the person who performs all of the following operations that are required to produce the product: (1) Mixing, (2) granulating, (3) milling, (4) molding, (5) lyophilizing, (6) tableting, (7) encapsulating, (8) coating, (9) sterilizing, and (10) filling sterile, aerosol, or gaseous drugs into dispensing containers.

(c) If no person performs all of the applicable operations listed in paragraph (b) of this section, no person may be represented as manufacturer except as follows:

(1) If the person performs more than one half of the applicable operations listed in paragraph (b) of this section and acknowledges the contribution of other persons who have performed the remaining applicable operations by stating on the product label that "Certain manufacturing operations have been performed by other firms."; or

(2) If the person performs at least one applicable operation listed in paragraph (b) of this section and identifies by appropriate designation all other persons who have performed the remaining applicable operations, e.g., "Made by (Person A), Filled by (Person B), Sterilized by (Person C)"; or

(3) If the person performs at least one applicable operation listed in paragraph (b) of this section and the person is listed along with all other persons who have performed the remaining applicable operations as "joint manufacturers." A list of joint manufacturers shall be qualified by the phrase "Jointly Manufactured By _____," and the names of all of the manufacturers shall be printed together in the same type size and style; or

(4) If the person performs all applicable operations listed in paragraph (b) of this section except for those operations listed in paragraph (d) of this section. For purposes of this paragraph, person, when it identifies a corporation, includes a parent, subsidiary, or affiliate company where the related companies are under common ownership and control.

(d) The Food and Drug Administration finds that it is the common practice in the drug industry to contract out the performance of certain manufacturing operations listed in paragraph (b) of this section. These operations include: (1) Soft-gelatin encapsulating, (2) aerosol filling, (3) sterilizing by irradiation, (4) lyophilizing, and (5) ethylene oxide sterilization.

(e) A person performs an operation listed in paragraph (b) of this section only if the operation is performed, including the performance of the appropriate in-process quality control operations, except laboratory testing of samples taken during processing, as follows:

(1) By individuals, a majority of whom are employees of the person and, throughout the performance of the operation, are subject to the person's direction and control;

(2) On premises that are continuously owned or leased by the person and subject to the person's direction and control; and

(3) On equipment that is continuously owned or leased by the person. As used in this paragraph, person, when it identifies a corporation, includes a parent, subsidiary, or affiliate company where the related companies are under common ownership and control.

(f) The name of the person represented as manufacturer under paragraph (b) or (c) of this section must be the same as either (1) the name of the establishment (as defined in § 207.3(b) of this chapter) under which that person is registered at the time the labeled product is produced or (2) the registered establishment name of a parent, subsidiary, or affiliate company where the related companies are under common ownership and control. In addition, the name shall meet the requirements of paragraph (g) of this section.

(g) The requirement for declaration of the name of the manufacturer, packer, or distributor shall be deemed to be satisfied, in the case of a corporate person, only by the actual corporate name, except that the corporate name may be the name of a parent, subsidiary, or affiliate company where the related companies are under common ownership and control. The corporate name may be preceded or followed by the name of the particular division of the corporation. "Company," "Incorporated," etc., may be abbreviated or omitted and "The" may be omitted. In the case of an individual, partnership, or association, the name under which the business is conducted shall be used.

(h)(1) Except as provided in this section, no person other than the manufacturer, packer, or distributor may be identified on the label of a drug or drug product.

(2) The appearance on a drug product label of a person's name without qualification is a representation that the named person is the sole manufacturer of the product. That representation is false and misleading, and the drug product is misbranded under section 502(a) of the act, if the person is not the manufacturer of the product in accordance with this section.

(3) If the names of two or more persons appear on the label of a drug or drug product, the label may identify which of the persons is to be contacted for further information about the product.

(4) If a trademark appears on the drug or drug product label or appears as a mark directly on the drug product (e.g., tablet or capsule), the label may identify the holder or licensee of the trademark. The label may also state whether the person identified holds the trademark or is licensee of the trademark.

(5) If the distributor is named on the label, the name shall be qualified by one of the following phrases: "Manufactured for _____". "Distributed by _____". "Manufactured by _____ for _____". "Manufactured for _____by _____". "Distributor: _____". "Marketed by _____". The qualifying phrases may be abbreviated.

(6) If the packer is identified on the label, the name shall be qualified by the phrase "Packed by _____" or "Packaged by _____". The qualifying phrases may be abbreviated.

(i) The statement of the place of business shall include the street address, city, State, and ZIP Code. For a foreign manufacturer, the statement of the place of business shall include the street address, city, country, and any applicable mailing code. The street address may be omitted if it is shown in a current city directory or telephone directory. The requirement for inclusion of the ZIP Code shall apply to consumer commodity labels developed or revised after July 1, 1969. In the case of nonconsumer packages, the ZIP Code shall appear either on the label or the labeling (including the invoice).

(j) If a person manufactures, packs, or distributes a drug or drug product at a place other than the person's principal place of business, the label may state the principal place of business in lieu of the actual place where such drug or drug product was manufactured or packed or is to be distributed, unless such statement would be misleading.

(k) Paragraphs (b), (c), (d), (e), and (f) of this section, do not apply to the labeling of drug components.

(l) A drug product is misbranded under section 502(a) of the act if its labeling identifies a person as manufacturer, packer, or distributor, and that identification does not meet the requirements of this section.

(m) This section does not apply to biological drug products that are subject to the requirements of section 351 of the Public Health Service Act, 42 U.S.C. 262.

[45 FR 25775, Apr. 15, 1980; 45 FR 72118, Oct. 31, 1980, as amended at 48 FR 37620, Aug. 19, 1983]

§ 201.2 Drugs and devices; National Drug Code numbers.

The National Drug Code (NDC) number is requested but not required to appear on all drug labels and in all drug labeling, including the label of any prescription drug container furnished to a consumer. If the NDC number is shown on a drug label, it shall be displayed as required in § 207.35(b)(3) of this chapter.

[40 FR 52002, Nov. 7, 1975]

§ 201.5 Drugs; adequate directions for use.

Adequate directions for use means directions under which the layman can use a drug safely and for the purposes for which it is intended. (Section 201.128 defines "intended use.") Directions for use may be inadequate because, among other reasons, of omission, in whole or in part, or incorrect specification of:

(a) Statements of all conditions, purposes, or uses for which such drug is intended, including conditions, purposes, or uses for which it is prescribed, recommended, or suggested in its oral, written, printed, or graphic advertising, and conditions, purposes, or uses for which the drug is commonly used; except that such statements shall not refer to conditions, uses, or purposes for which the drug can be safely used only under the supervision of a practitioner licensed by law and for which it is advertised solely to such practitioner.

(b) Quantity of dose, including usual quantities for each of the uses for which it is intended and usual quantities for persons of different ages and different physical conditions.

(c) Frequency of administration or application.

(d) Duration of administration or application.

(e) Time of administration or application (in relation to time of meals,

time of onset of symptoms, or other time factors).

(f) Route or method of administration or application.

(g) Preparation for use, i.e., shaking, dilution, adjustment of temperature, or, other manipulation or process.

[41 FR 6908, Feb. 13, 1976]

§ 201.6 Drugs; misleading statements.

(a) Among representations in the labeling of a drug which render such drug misbranded is a false or misleading representation with respect to another drug or a device or a food or cosmetic.

(b) The labeling of a drug which contains two or more ingredients may be misleading by reason, among other reasons, of the designation of such drug in such labeling by a name which includes or suggests the name of one or more but not all such ingredients, even though the names of all such ingredients are stated elsewhere in the labeling.

[41 FR 6908, Feb. 13, 1976]

§ 201.10 Drugs; statement of ingredients.

(a) The ingredient information required by section 502(e) of the Federal Food, Drug, and Cosmetic Act shall appear together, without any intervening written, printed, or graphic matter, except the proprietary names of ingredients, which may be included with the listing of established names, and such statements as "Warning—May be habit forming" that are specifically required for certain ingredients by the act or regulations in this chapter.

(b) The term *ingredient* applies to any substance in the drug, whether added to the formulation as a single substance or in admixture with other substances.

(c) The labeling of a drug may be misleading by reason (among other reasons) of:

(1) The order in which the names of the ingredients present in the drug appear in the labeling, or the relative prominence otherwise given such names.

(2) Failure to reveal the proportion of, or other fact with respect to, an ingredient present in such drug, when such proportion or other fact is material in the light of the representation that such ingredient is present in such drug.

(3) The employment of a fanciful proprietary name for a drug or ingredient in such a manner as to imply that the drug or ingredient has some unique effectiveness or composition when, in fact, the drug or ingredient is a common substance, the limitations of which are readily recognized when the drug or ingredient is listed by its established name.

(4) The featuring in the labeling of inert or inactive ingredients in a manner that creates an impression of value greater than their true functional role in the formulation.

(5) Designation of a drug or ingredient by a proprietary name that, because of similarity in spelling or pronunciation, may be confused with the proprietary name or the established name of a different drug or ingredient.

(d)(1) If the drug is in tablet or capsule form or other unit dosage form, any statement of the quantity of an ingredient contained therein shall express the quantity of such ingredient in each such unit. If the drug is not in unit dosage form, any statement of the quantity of an ingredient contained therein shall express the amount of such ingredient in a specified unit of weight or measure of the drug, or the percentage of such ingredient in such drug. Such statements shall be in terms that are informative to licensed practitioners, in the case of a prescription drug, and to the layman, in the case of a nonprescription drug.

(2) A statement of the percentage of an ingredient in a drug shall, if the term *percent* is used without qualification, mean percent weight-in-weight, if the ingredient and the drug are both solids, or if the ingredient is a liquid and the drug is a solid; percent weight in volume at 68 °F. (20 °C.), if the ingredient is a solid and the drug is a liquid; and percent volume in volume at 68 °F. (20 °C.), if both the ingredient and the drug are liquids, except that alcohol shall be stated in terms of percent volume of absolute alcohol at 60 °F. (15.56 °C.).

(e) A derivative or preparation of a substance named in section 502(e) of the act is an article derived or prepared

from such substance by any method, including actual or theoretical chemical action.

(f) If an ingredient is a derivative or preparation of a substance specifically named in section 502(e) of the act and the established name of such ingredient does not indicate that it is a derivative or preparation of the parent substance named in section 502(e) of the act, the labeling shall, in conjunction with the listing of the established name of such ingredient, declare that such article is a derivative or preparation of such parent substance.

(g)(1) If the label or labeling of a prescription drug bears a proprietary name or designation for the drug or any ingredient thereof, the established name, if such there be, corresponding to such proprietary name or designation shall accompany such proprietary name or designation each time it is featured on the label or in the labeling for the drug; but, except as provided in this subparagraph, the established name need not be used with the proprietary name or designation in the running text of the label or labeling. On any label or page of labeling in which the proprietary name or designation is not featured but is used in the running text, the established name shall be used at least once in the running text in association with such proprietary name or designation and in the same type size used in such running text: *Provided, however,* That if the proprietary name or designation is used in the running text in larger size type, the established name shall be used at least once in association with, and in type at least half as large as the type used for, the most prominent presentation of the proprietary name or designation in such running text. If any labeling includes a column with running text containing detailed information as to composition, prescribing, side effects, or contraindications and the proprietary name or designation is used in such column but is not featured above or below the column, the established name shall be used at least once in such column of running text in association with such proprietary name or designation and in the same type size used in such column of running text: *Provided, however,* That if the proprietary name or designation is used in such column of running text in larger size type, the established name shall be used at least once in association with, and in type at least half as large as the type used for, the most prominent presentation of the proprietary name or designation in such column of running text. Where the established name is required to accompany or to be used in association with the proprietary name or designation, the established name shall be placed in direct conjunction with the proprietary name or designation, and the relationship between the proprietary name or designation and the established name shall be made clear by use of a phrase such as "brand of" preceding the established name, by brackets surrounding the established name, or by other suitable means.

(2) The established name shall be printed in letters that are at least half as large as the letters comprising the proprietary name or designation with which it is joined, and the established name shall have a prominence commensurate with the prominence with which such proprietary name or designation appears, taking into account all pertinent factors, including typography, layout, contrast, and other printing features.

(h)(1) In the case of a prescription drug containing two or more active ingredients, if the label bears a proprietary name or designation for such mixture and there is no established name corresponding to such proprietary name or designation, the quantitative ingredient information required on the label by section 502(e) of the act shall be placed in direct conjunction with the most prominent display of the proprietary name or designation. The prominence of the quantitative ingredient information shall bear a reasonable relationship to the prominence of the proprietary name.

(2) If the drug is packaged in a container too small to bear the quantitative ingredient information on the main display panel, the quantitative ingredient information required by section 502(e) of the act may appear elsewhere on the label, even though the proprietary name or designation appears on the main display panel of the

label; but side- or back-panel placement shall in this case be so arranged and printed as to provide size and prominence of display reasonably related to the size and prominence of the front-panel display.

(i) A drug packaged in a container too small or otherwise unable to accommodate a label with sufficient space to bear the information required for compliance with section 502(e)(1)(A)(ii) and (B) of the act shall be exempt from compliance with those clauses: *Provided*, That:

(1) The label bears:

(i) The proprietary name of the drug;

(ii) The established name, if such there be, of the drug;

(iii) An identifying lot or control number; and

(iv) The name of the manufacturer, packer, or distributor of the drug; and

(2) All the information required to appear on the label by the act and the regulations in this chapter appears on the carton or other outer container or wrapper if such carton, outer container, or wrapper has sufficient space to bear such information, or such complete label information appears on a leaflet with the package.

§ 201.15 Drugs; prominence of required label statements.

(a) A word, statement, or other information required by or under authority of the act to appear on the label may lack that prominence and conspicuousness required by section 502(c) of the act by reason, among other reasons, of:

(1) The failure of such word, statement, or information to appear on the part or panel of the label which is presented or displayed under customary conditions of purchase;

(2) The failure of such word, statement, or information to appear on two or more parts or panels of the label, each of which has sufficient space therefor, and each of which is so designed as to render it likely to be, under customary conditions of purchase, the part or panel displayed;

(3) The failure of the label to extend over the area of the container or package available for such extension, so as to provide sufficient label space for the prominent placing of such word, statement, or information;

(4) Insufficiency of label space for the prominent placing of such word, statement, or information, resulting from the use of label space for any word, statement, design, or device which is not required by or under authority of the act to appear on the label;

(5) Insufficiency of label space for the prominent placing of such word, statement, or information, resulting from the use of label space to give materially greater conspicuousness to any other word, statement, or information, or to any design or device; or

(6) Smallness or style of type in which such word, statement, or information appears, insufficient background contrast, obscuring designs or vignettes, or crowding with other written, printed, or graphic matter.

(b) No exemption depending on insufficiency of label space, as prescribed in regulations promulgated under section 502 (b) or (e) of the act, shall apply if such insufficiency is caused by:

(1) The use of label space for any word, statement, design, or device which is not required by or under authority of the act to appear on the label;

(2) The use of label space to give greater conspicuousness to any word, statement, or other information than is required by section 502(c) of the act; or

(3) The use of label space for any representation in a foreign language.

(c)(1) All words, statements, and other information required by or under authority of the act to appear on the label or labeling shall appear thereon in the English language: *Provided, however*, That in the case of articles distributed solely in the Commonwealth of Puerto Rico or in a Territory where the predominant language is one other than English, the predominant language may be substituted for English.

(2) If the label contains any representation in a foreign language, all words, statements, and other information required by or under authority of the act to appear on the label shall appear thereon in the foreign language.

(3) If the labeling contains any representation in a foreign language, all words, statements, and other information required by or under authority of

article unless the dosage form is labeled as required by section 503(b) and §§ 201.100 or 201.105.

[41 FR 6911, Feb. 13, 1976]

§ 201.128 Meaning of "intended uses".

The words *intended uses* or words of similar import in §§ 201.5, 201.115, 201.117, 201.119, 201.120, and 201.122 refer to the objective intent of the persons legally responsible for the labeling of drugs. The intent is determined by such persons' expressions or may be shown by the circumstances surrounding the distribution of the article. This objective intent may, for example, be shown by labeling claims, advertising matter, or oral or written statements by such persons or their representatives. It may be shown by the circumstances that the article is, with the knowledge of such persons or their representatives, offered and used for a purpose for which it is neither labeled nor advertised. The intended uses of an article may change after it has been introduced into interstate commerce by its manufacturer. If, for example, a packer, distributor, or seller intends an article for different uses than those intended by the person from whom he received the drug, such packer, distributor, or seller is required to supply adequate labeling in accordance with the new intended uses. But if a manufacturer knows, or has knowledge of facts that would give him notice, that a drug introduced into interstate commerce by him is to be used for conditions, purposes, or uses other than the ones for which he offers it, he is required to provide adequate labeling for such a drug which accords with such other uses to which the article is to be put.

[41 FR 6911, Feb. 13, 1976]

§ 201.129 Drugs; exemption for radioactive drugs for research use.

A radioactive drug intended for administration to human research subjects during the course of a research project intended to obtain basic research information regarding metabolism (including kinetics, distribution, and localization) of a radioactively labeled drug or regarding human physiology, pathophysiology, or biochemistry (but not intended for immediate therapeutic, diagnostic, or similar purposes), under the conditions set forth in § 361.1 of this chapter, shall be exempt from section 502(f)(1) of the act if the packaging, label, and labeling are in compliance with § 361.1(f) of this chapter.

[41 FR 6911, Feb. 13, 1976]

Subpart E—Other Exemptions

§ 201.150 Drugs; processing, labeling, or repacking.

(a) Except as provided by paragraphs (b) and (c) of this section, a shipment or other delivery of a drug which is, in accordance with the practice of the trade, to be processed, labeled, or repacked in substantial quantity at an establishment other than that where originally processed or packed, shall be exempt, during the time of introduction into and movement in interstate commerce and the time of holding in such establishment, from compliance with the labeling and packaging requirements of sections 501(b) and 502 (b), (d), (e), (f), and (g) of the act if:

(1) The person who introduced such shipment or delivery into interstate commerce is the operator of the establishment where such drug is to be processed, labeled, or repacked; or

(2) In case such person is not such operator, such shipment or delivery is made to such establishment under a written agreement, signed by and containing the post-office addresses of such person and such operator, and containing such specifications for the processing, labeling, or repacking, as the case may be, of such drug in such establishment as will insure, if such specifications are followed, that such drug will not be adulterated or misbranded within the meaning of the act upon completion of such processing, labeling, or repacking. Such person and such operator shall each keep a copy of such agreement until 2 years after the final shipment or delivery of such drug from such establishment, and shall make such copies available for inspection at any reasonable hour to any officer or employee of the Department who requests them.

Exhibit K

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jnm/ RADIOCHEMISTRY AND RADIOPHARMACY

IODINE-123-ROSE BENGAL: AN IMPROVED HEPATOBILIARY IMAGING AGENT

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A practical method for preparing ^{123}I-rose bengal that allows for its rapid and efficient incorporation into the molecule is reported. Administration of ^{123}I-rose bengal to normal healthy patients showing the normal uptake and excretory pattern visualized with this radiopharmaceutical is also presented. The overall reduction in imaging time and radiation exposure together with the improved images possible should greatly improve our diagnostic capabilities in evaluating the jaundiced patient.

Iodine-131-rose bengal was first developed for use as a test of liver function by Taplin, et al in 1955 (*1*). Since then the ^{131}I-rose bengal test in conjunction with other liver function tests has been shown to be useful in evaluating the jaundiced patient by a number of investigators (*2–4*). The technique currently utilizes dynamic scintigraphy with the Anger scintillation camera as described by Burke and Halko (*5*) in which the visual delineation of both hepatic uptake and excretion of the radiolabeled dye and the time sequence of their occurrence can be assessed. This is usually combined with a blood pool clearance study performed by placing a scintillation detector against the side of the head so that a 20-min retention can be measured. Analysis of these data has been found to correlate with the clearance of the radiopharmaceutical from the blood (*6*).

Because of the relatively high absorbed radiation dose produced by the beta decay and the 8-day half-life of ^{131}I-rose bengal, we are limited in the amount of activity that can be administered to the patient (usually 200–300 μCi). The low photon flux with low count statistics results in a relatively prolonged imaging time or poor resolution image. The problems are often aggravated by the poor functional capacity of the hepatobiliary system in the presence of disease. With the availability of large quantities of cyclotron-produced ^{123}I at our institution, the feasibility of developing a new formulation of rose bengal was investigated.

The preparation of the ^{123}I-labeled rose bengal and its distribution in animals was recently reported (*7*). This has since led us to investigate further and evaluate this radiopharmaceutical in humans. This study outlines some of our preliminary findings.

MATERIALS AND METHODS

The ^{123}I used in this study was produced on the Mount Sinai Medical Center cyclotron using the ^{124}Te(p,2n)^{123}I reaction. Details regarding the production, chemical separation, and radionuclidic purity have been published elsewhere (*8,9*).

Chemically, rose bengal is a halogenated fluorescein dye, principally tetrachlorotetraiodofluorescein. Commercially available nonradioactive rose bengal may contain a number of related hologenated fluoresceins that may or may not be handled similarly by the liver (*10,11*). In order to reduce variations possibly resulting from the starting material, we tested the rose bengal of three commercial suppliers (Eastman, K&K, and Fisher) for chemical homogeneity. Ten milligrams of rose bengal from each supplier were dissolved in isotonic sodium chloride solution and applied separately to a 37- × 2-cm column of Bio-Gel P-2. The rose bengal was slowly eluted with saline causing a separation of various components of the dye identifiable by visible and ultraviolet light spectroscopy. The rose bengal from two of the suppliers contained numerous components

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while that from the third (Fisher) contained three, two of which were capable of being tagged by our iodination procedure. The major component, having about 95% of the tagged radioactivity, has an absorption maxima at 550 mμ corresponding to 4,5,6,7 tetrachloro-2'4'5'7' tetraiodofluorescein (*12*). The rose bengal from Fisher Scientific was used for our studies.

Iodination of the rose bengal is performed by refluxing a mixture of 10–50 mg rose bengal, 10–50 mCi of ^{123}I, and 0.15 ml hydrogen peroxide contained in a maximum of 10 ml acetate buffer at pH 5.2 for 1 hr. Just prior to the addition to the reaction vessel of the ^{123}I contained in a maximum volume of 4 ml, the pH is adjusted to 2 followed by the addition of 0.25 mg of sodium iodide per milliliter of ^{123}I solution. Following reflux, the unbound radioactivity is converted to iodide by the addition of 50 mg sodium thiosulfate and is separated by precipitating the rose bengal with 2 ml of 6 *M* HCl and filtering the supernate. The precipitate is washed with 100 ml of 0.01 *M* HCl followed by sterile water until the rose bengal begins to dissolve as evidenced by a slight pink tint in the wash water. The iodinated rose bengal is dissolved in phosphate buffer, pH 7.2, filtered and adjusted with sterile diluent to contain 1.8–2.2 mg rose bengal/ml, 0.9–1.1 mCi ^{123}I/ml and pH 7–7.5. The yields of the iodination procedure range between 65 and 75%.

Radiochemical purity is determined by ascending chromotography in a 50:50 mixture of 5.8% ammonium hydroxide and ethanol using Whatman No. 1 paper. In this system, the R_f values for rose bengal and iodide are 0.43 and 0.85, respectively. The final product is autoclaved at 121°C for 30 min and each batch is tested for sterility and pyrogens according to USP 18 procedures. The total time in preparing the radiopharmaceutical by this method is about 3 hr.

Seven normal healthy volunteers with no clinical or biochemical evidence of hepatobiliary disease were studied. Patients were prepared by having them fast from midnight the night before the exam. Each patient was injected intravenously with 2 mCi of ^{123}I-rose bengal. Blood clearance studies were performed in addition to measuring 24-hr urinary excretion. Simultaneously, sequential scintiphotos of the cardiac pool, liver, biliary system, and intestines were taken at various intervals throughout the study using the Anger scintillation camera with a high-energy parallel-hole collimator. Polaroid scintiphotos with 300,000 counts per view were taken. Utilizing an on-line computer, the data were also recorded and stored for subsequent playback analysis. Time–activity curves could thus be generated from various selected regions of interest that included the activity in the cardiac pool, liver, gallbladder, common bile duct, where visible, and in the gastrointestinal tract.

The estimated absorbed dose from our ^{123}I-rose bengal in rads per millicurie is as follows: liver, 0.2; gallbladder, 3.8; ovaries, 0.3; testes, 0.03; and total body, 0.08 (*13*).

RESULTS

The scintiphoto sequence of events recorded in two typical normal patients is shown in Figs. 1 and 2. Significant findings are that, within the first 30 min, sufficient localization of the radiopharmaceutical within the liver parenchymal cells is seen so that good anatomic delineation of the liver can be obtained.

Soon thereafter, two different patterns seem to emerge. First, there may be subsequent localization of the activity within the region of the gallbladder that increases with time. At a certain point, usually before maximal concentration in the gallbladder is reached, evacuation of the rose bengal into the gastrointestinal tract is visualized. This emptying is manifested by visible activity in the region of the common duct and subsequently the duodenum (Fig. 1). Pooling of activity within the region of the jejunal loop is then noted. In some instances, especially if the rate of emptying occurs early, the duct and duodenum are not well visualized and only activity within the proximal portion of the jejunem is seen initially. This may be visualized sometimes even before gallbladder filling occurs.

Subsequent progressive decline in activity within the liver with diminishing activity in the gallbladder is seen during the next 4–6 hr. At 24 hr only activity throughout the bowel is seen. The marked improvement in the quality of the images is apparent. Liver images approaching the quality obtained with ^{99m}Tc-sulfur colloid were observed in most instances. Marked improvement in the anatomic detail of the biliary tree was evident with excretion of the ^{123}I-rose bengal into the gastrointestinal tract being readily visible. The overall imaging time to obtain the 300,000 counts per scintiphoto varied between 90–360 sec depending on the time after injection the scintiphoto was obtained. This is a considerable reduction in time when compared with ^{131}I-rose bengal.

To ascertain that the ^{123}I-rose bengal was stable in vivo, sequential whole-body scans to encompass the thyroid and stomach were performed. The whole-body scans showed that after injection, the majority of the radioactivity was concentrated in the liver and the amount then decreased sharply with a concomitant increase in the intestinal tract. After 24 hr some thyroid uptake was observed suggesting that some iodine is slowly released from the rose bengal

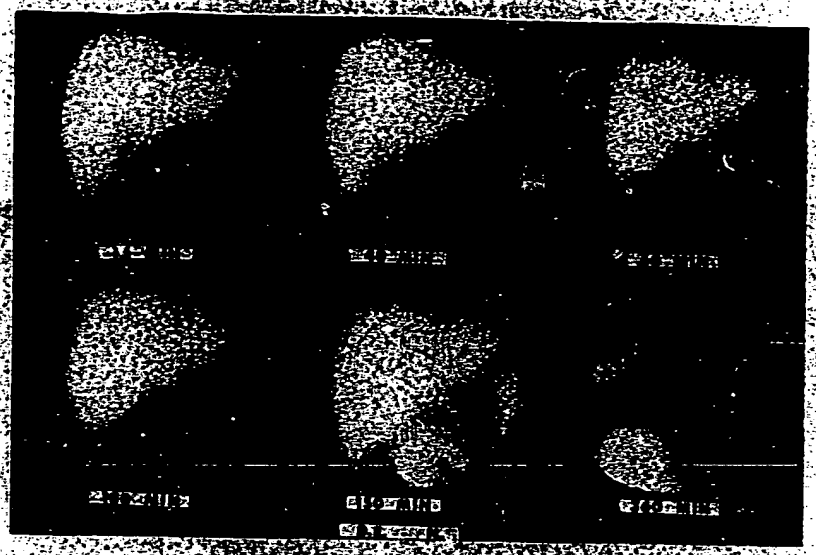


FIG. 1. Sequence of events demonstrating initial uptake of ^{123}I-rose bengal into parenchymal cells with subsequent visualization of common duct at 60 min and excretion into second portion of duodenum at 100 min. Note that excretion is occurring prior to complete filling of gallbladder.

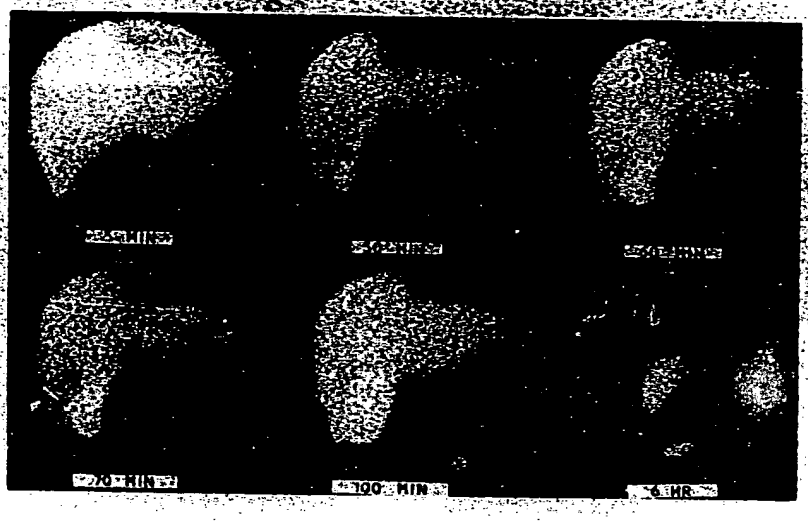


FIG. 2. Sequence of events demonstrating initial uptake of ^{123}I-rose bengal into parenchymal cells with subsequent visualization of gallbladder. Activity in common duct or duodenum is not seen; however, excretion is appreciated by activity present in left upper quadrant in proximal portion of duodenum.

molecule in vivo probably due to metabolism. The blood clearance during the first 30 min is demonstrated in Fig. 3 and is comparable to the results reported with ^{131}I-rose bengal (*6*). The 24-hr urinary excretion in this group averaged 2% of the administered activity.

DISCUSSION

Iodine-123 when compared with ^{131}I has several desirable physical properties that have encouraged us to substitute ^{123}I as a label for radiopharmaceuticals (*14,15*). The major deterrent to the use of ^{123}I has been lack of availability in sufficient quantities for routine clinical use. Hundred-millicurie amounts of ^{123}I have been produced at the Mount Sinai Medical Center cyclotron from an enriched tellurium target with a radionuclidic purity of 99.1% at the end of bombardment. With the availability of these high yields, millicurie quantities of high specific activity radiopharmaceuticals become possible. The

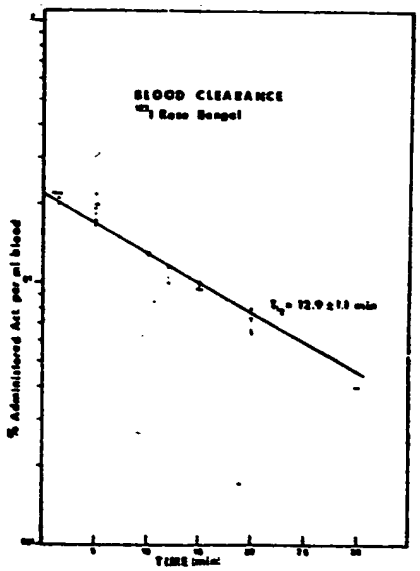


FIG. 3. Blood clearance of ^{123}I-rose bengal in normal patients during first 30 min.

relatively short 13-hr half-life of ^{123}I decreases patient exposure, and administration of millicurie quantities for diagnostic studies becomes feasible. However, rapid and efficient incorporation of ^{123}I in the molecule becomes more important than with 8-day half-life ^{131}I analogs. We have developed an expeditious method of routinely preparing high specific activity ^{123}I-rose bengal. Administration of ^{123}I-rose bengal to normal healthy volunteers vividly demonstrates the functioning anatomy of the liver, gallbladder, and biliary tree. A practical method for preparing large quantities of ^{123}I, considerations in selection of a rose bengal dye, the technique used for formulating the radiopharmaceutical, and distribution studies are reported in this study.

Based on these results, we feel that ^{123}I-rose bengal will prove advantageous in the evaluation of hepatobiliary disorders especially in the jaundiced patient in whom the differentiation between hepatocellular and extrahepatic obstructive jaundice may not be apparent from the biochemical and clinical presentation.

ACKNOWLEDGMENT

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REVIEW

ROSE BENGAL†

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Summary

The spectral properties, photochemical reactivity and photophysical parameters of all of the known derivatives of Rose Bengal, 2,4,5,7-tetraiodo-3,'4',5',6'-tetrachlorofluorescein, are reported and compared.

1. Background

Rose Bengal, 2,4,5,7-tetraiodo-3,'4',5',6'-tetrachlorofluorescein [1], was originally synthesized by Gnehm [2] as a fabric dye to mimic the red colors in "bengalis" [3]. Its name is connected to the red symbolic spot worn at the part of the hair by Bengali women to symbolize marriage [4]. Much of the history of Rose Bengal has been reviewed elsewhere [5, 6].

Rose Bengal, disodium salt, holds an important position among all dyes for a number of reasons:

1. It has a large absorption in all solvents in which it is soluble which corresponds almost exactly to the sodium D line.
2. Its intersystem crossing yield is high, but Φ_{isc} is not unity. All Rose Bengals show some fluorescence.
3. Its triplet is completely quenched by oxygen.
4. Its spectrum is most diagnostic of its immediate environment.
5. It bleaches slowly in protic, polar solvents.
6. It is a photodynamic sensitizer.
7. Its singlet may be quenched by strong oxidizing agents, in some cases forming radicals.

The purpose of this review is to summarize the spectral, photophysical, and photochemical properties of Rose Bengal, disodium salt, as well as those of its many derivatives. We refer to the disodium salt of 2,4,5,7-tetraiodo-3,'4',5',6'-tetrachlorofluorescein as Rose Bengal since this was Gnehm's original dyestuff and it is what is sold commercially as Rose Bengal.

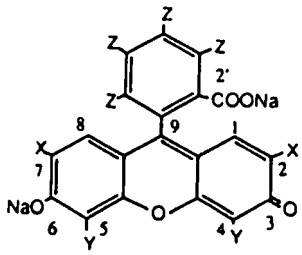
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TABLE 1

The xanthenes



Compound	*X*	*Y*	*Z*	λ_{max} (nm) *in H_2O*	λ_{max} (nm) *in EtOH*	ϕ(EtOH)[a]	ϕ(H_2O)[a]	pK_1[b]	pK_2
Rose Bengal	I	I	Cl	549.0	557.0	0.86	0.75	3.72	—
Phloxin	Br	Br	Cl	538.5	547.5	0.40	0.65	3.69	—
Erythrosin	I	I	H	526.5	532	0.69	0.63	4.18	—
Eosin	Br	Br	H	517.0	523	0.32	0.57	3.80	—
	Cl	Cl	H	510.0	520	0.05	0.05	—	
	I	H	H	506.5	513	0.33	0.48	3.75	4.76
	Br	H	H	504	510	0.32	0.42	—	
	H	Cl	H	502.5	511	0.04	0.07	—	
	Cl	H	H	502.5	511	0.04	0.07	3.69	4.91
Fluorescein	H	H	H	490.0	499	0.03	0.03	4.38	6.99

Most of the data in this table is from E. Gandin, Y. Lion and A. Van de Vorst, *Photochem. Photobiol.*, *37* (1983) 271.

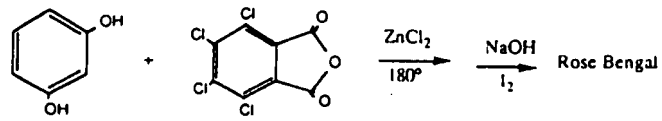
[a] Singlet oxygen formation.

[b] Data from I. M. Issa, R. M. Issa and M. M. Ghoneim, *Z. Phys. Chem.*, *250* (1922) 161.

For many of the other xanthenes, Eosin and fluorescein in particular, ring-substituted derivatives are also referred to as Eosins or fluoresceins respectively (for example Eosin B or fluorescein yellow). In general these compounds have the same ring skeleton as the parent compound but completely different ring substituents. To avoid confusion we will draw structures where necessary. Though we will generally avoid detailed comparisons with the xanthenes as a group, comparisons with fluorescein, Erythrosin and Eosin will be made where appropriate.

2. Analogs and synthesis

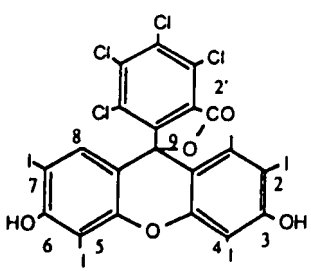
The structures and simple photophysical properties of the dyes in water and EtOH are shown in Table 1. The synthesis of the xanthenes is from resorcinol and a phthalic anhydride, in the case of Rose Bengal tetrachlorophthalic anhydride. It is trivial but messy.



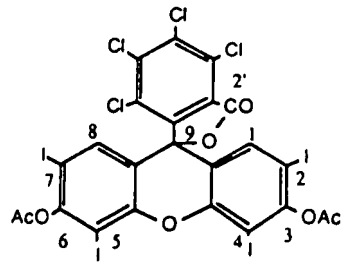
resorcinol

As a dye neither Rose Bengal nor any of its derivatives has current commercial application though Erythrosin is used as the red dye in marachino cherries and Tylenol® capsules. Eosin has many biological uses, as a biological stain for eosinophiles, for example. The uses of fluorescein derivatives are unique and extremely important. It is used as a diagnostic probe in many clinical applications, and in fact there is even a company which markets such compounds called Molecular Probes. Unfortunately these applications are extensive and would require much more space than is allowed for this review. We will confine our report to the chemistry of the Rose Bengals and discuss biological issues in a subsequent review.

The disodium salt of Rose Bengal is difficult to purify and the commercially available dyes are not seriously purified after synthesis. Rose Bengal frequently contains inorganic salts either derived during the synthesis or added as part of an old farbstoff protocol. Dye purity is reported relative to Colour Index methods [7, 8] which are often old [5]. Rose Bengal has been reportedly purified by chromatography [9] though these methods often accomplish little other than making the laboratory table a big red mess. Rose Bengal is best purified by conversion to the lactone [10] or to the lactone diacetate [11], with subsequent recrystallization and stoichiometric reconversion to the disodium salt.



lactone



lactone diacetate

The C-2',C-6 ammonium salts [10], phosphonium salts, iodonium salts and other organic derivatives are easily prepared from the lactone by neutralization with the appropriate Lewis base. These organic ion salts are much easier to purify though they are not, for the most part, soluble in water.

3. Absorption and emission spectra

Rose Bengals retain their general xanthene color and spectra if ionization at C-6 is supported by the solvent. This depends on a number of factors,

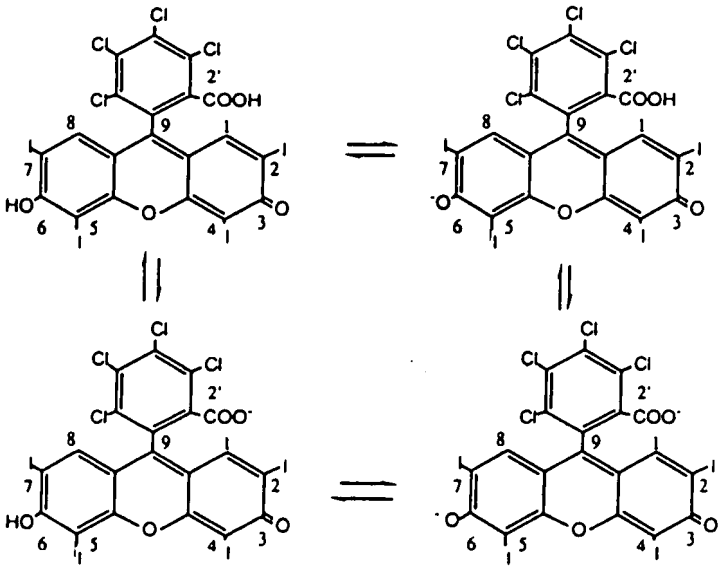


Fig. 1. Top left, C-2′(H),C-6(H); top right, C-6 monoanion; bottom left, C-2′ monoanion; C-6(H); bottom right, dianion.

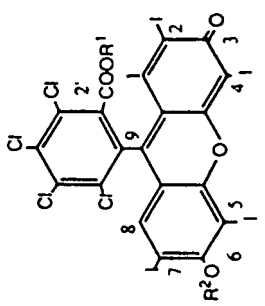
among which is the solubility of the gegen ion in the supporting solvent. The essential equilibria are shown in Fig. 1. The important forms of Rose Bengal and all of the xanthenes are the C-6 monoanion (Fig. 1, top right) and C-2′,C-6 dianion (Fig. 1, bottom right). The fully protonated C-2′(H), C-6(H) (Fig. 1, top left) has never been observed since it undergoes an immediate ring closure to the lactone. The pK_1 of the C-6 protio form of Rose Bengal has been measured in water as 3.72. This contrasts with a value for the ionization constant for the pK_2 at C-2′ of 4.75 for diiodofluorescein (Table 1). The ionization constant at C-2′ in Rose Bengal has not been (or cannot be) measured because ring closure occurs to the lactone immediately upon formation of the C-2′ acid.

Essentially three different kinds of Rose Bengal derivatives have been synthesized. Salts with gegen ions other than sodium at both C-2′ and C-6,C-2′ ester, C-6 salts, and C-2′ ester, C-6 ethers. The absorption spectra of all the known Rose Bengal dianionic and C-2′ ester, C-6 monoanions are given in Table 2. The emission spectra of known Rose Bengal derivatives are given in Table 3.

Four variables cause significant changes in the spectrum of the dye: (a) C-6 effects and ionization at C-6 [12]; (b) C-2′ substitution; (c) solvent and solvent composition and (d) concentration. We discuss each of these issues separately below.

TABLE 2

Absorption properties of known derivatives of Rose Bengal



Compound	Water[†]		MeOH		EtOH		CH_2Cl_2		DME		MeTHF	Other	
$R^1 = R^2 = Na^+$	515	549	518	558	515 (32500)	557 (105000)	–	–	518 (31200)	557 (82200)	–	n-PrOH (559)[a]	n-BuOH (561)[a]
Li^+, Li^+[b]	–	–	–	–	519 (37900)	559 (121000)	–	–	–	–	–	–	–
K^+, K^+[b]	–	–	–	–	520 (36000)	558 (120000)	–	–	–	–	–	–	–
Cs^+, Cs^+[b]	–	–	–	–	519 (47280)	559 (148000)	–	–	–	–	–	–	–
$\phi_3P^+CH_3, \phi_3P^+CH_3$[c]	–	–	–	–	519 (35600)	558 (118000)	522 (37500)	566 (102200)	524 (33000)	565 (84300)	–	–	
$\phi_3P^+CH_3;$ $\phi_3P^+CH_3$[c]	–	–	–	–	518 (34100)	559 (115900)	529 (29700)	564 (114200)	520 (29500)	563 (110700)			
$(CH_3)_2NH^+;$ $(CH_3)_2NH^+$[b]	–	–	–	–	519.5 (38700)	559 (117000)	523 (27600)	557 (59600)	–	–	–	–	–
$(Et)_2NH^+;$ $(Et)_2NH^+$[b]	–	–	–	–	521 (33200)	559 (117000)	519 (44900)	556 (101500)	–	–	–	–	–

(continued)

TABLE 2. *(continued)*

Compound	Water[†]		MeOH		EtOH		CH_2Cl_2		DME		MeTHF	Other	
$(Et)_3NH_2^+$; $(Et)_3NH_2^{+b}$	–	–	–	–	520 (29100)	558.5 (115000)	527[‡]	553	–	–	–	–	–
piperidine; piperidine[b]	–	–	–	–	521 (43700)	559 (122000)	526[‡]	553	–	–	–	–	–
$R^1 = CH_3$; $R^2 = Na^+$		548 (113600)	–	–	527 (37100)	568.5 (106000)	–	–	–	–	–	–	–
$R^1 = C_2H_5$; $R^2 = Na^+$	–	–	–	–	524 (29600)	565 (105300)	–	–	–	–	–	–	–
$R^1 = CH_2\phi$; $R^2 = Na^{+b,d}$	–	–	–	–	527 (33800)	568.2 (95800)	– –	– –	528 (39300)	569 (99400)	–	–	–
$R^1 = CH_3$; $R^2 = H^{+b}$	–	–	–	–	526 (40900)	566 (117000)	–	–	–	–	–	–	–
$R^1 = CH_2\phi$; $R^2 = TMA^{+b}$	–	–	–	–	527 (26000)	564 (73600)	527 (30600)	566 (45700)	–	–	–	–	–
$R^1 = CH_2\phi$; $R^2 = TEA^{+b}$	–	–	–	–	527 (36200)	563.5 (93200)	527 (24600)	564 (44300)	–	–	–	–	–
$R^1 = CH_2\phi$; $R^2 = DEA^{+b}$	–	–	–	–	527 (38500)	563 (104000)	530 (35500)	561 (47600)	–	–	–	–	–
R^1 = octyl; $R^2 = (Bu)_4N^{+e}$	–	–	525	564	524 (24050)	565 (72070)	–	–	–	–	–	toluene 536 563	
R^1 = octyl; $R^2 = (Bu)_3NH^+$	–	–	–	–	488 (3500)	525 (40500)	567 (128000)	–	–	–	–	toluene 536 563 537 (34000) 585 (65000)[g]	
R^1 = ethyl; $R^2 = Et_3NH^{+e}$	–	–	524	563	–	–	525	563	–	–	–	–	–
R^1 = ethyl; $R^2 = Bu_4N^{+e}$	–	–	523	563	527	566	532	574	–	–	–	–	toluene 537 570
$R^1 = CH_2\phi$; R^2 = piper^{+b}	–	–	–	–	528 (38400)	565.7 (94400)	526.5 (41000)	565.7 (42700)	–	–	–	–	–
$R^1 = CH_2\phi$; $R^2 = \phi_2I^{+c}$	–	–	–	–	526 (38300)	568 (119000)	530 (38100)	572 (92600)	530 (37300)	572 (92300)	–	toluene 540 (39400)	569 (43400)
$R^1 = CH_2\phi$; $R^2 = \phi_3PCH_3^{+c}$	–	–	–	–	525 (36100)	567 (118000)	530 (32700)	573 (118000)	528 (28400)	572 (96500)	–	toluene 534 (27500)	574 (59400)
$R^1 = CH_2\phi$; R^2 = triphenyl pyrilium^{+c}	–	–	–	–	527 (27500)	568 (864000)	528 (20800)	572 (52600)	525 (15200)	573 (17800)	–	toluene 526[‡]	570
$R^1 = CH_2\phi$; $R^2 = \phi_2SCH_3^{+c}$	–	–	–	–	525 (26300)	566 (80200)	529 (27600)	572 (92000)	529 (19500)	573 (50400)	–	toluene 530 (7300)	568 (6000)
R^1 = Z-Lys, TEA[b]	–	–	–	–	525 (11670)	564 (33400)	–	–	–	–	–	–	–
R^1 = Z-(Lys)$_4$-OMe; TEA^{+b}	–	–	–	–	525 (11740)	565 (33500)	–	–	–	–	–	–	–
$R^1 = -(CH_2)_5CO_2^-$ $R^2 = Na^{+e}$	–	551.5 (82100)	–	–	–	–	–	–	–	–	–	–	–
$R^1 = -(CH_2)_{10}CO_2^-$ $R^2 = Na^{+f}$	–	557 (79200)	–	–	–	–	–	–	–	–	–	–	–
$R^1 = -(CH_2)_{10}CO_2$-β-cyclodextrin $R^2 = Na^+$	–	562 (30200)	–	–	–	–	–	–	–	–	–	–	–
$R^1 = -(CH_2)_5$, $R^2 = Na^+, Na^+$	–	–	–	–	527 (66000)	565 (183400)	–	–	–	–	–	515 (46100)	547[g] (78900)
$R^1 = -(CH_2)_{10}$; $R^2 = Na^+, Na^+$	–	–	–	–	480 (12300)	526 (63200)	565 (190200)	–	–	–	–	510 (26700)	546[g] (48400)

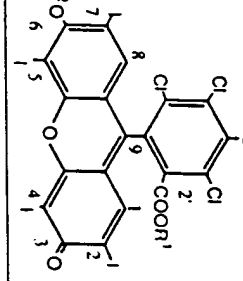
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TABLE 2 *(continued)*

Compound	*Water*[†]		*MeOH*		*EtOH*		*CH_2Cl_2*		*DME*		*MeTHF*	*Other*	
R^1 = $-(CH_2)_{16}$; R^2 = Na^+, Na^+	–	–	–	–	485 (13200)	520 (61100)	565 (20000)	–	–	–	–	511 (50400)	544 (98800)
R^1 = $-(CH_2)_5$; R^2 = NBu_4^+; NBu_4^+	–	–	–	–	483 (12900)	523 (57900)	561 (16800)	–	–	–	–	–	–
R^1 = polystyrene; R^2 = Na^+;	–	–	–	–	–	–	–	523	562			529 534 534	572[h] 562[i] 562[j]

[†] All entries λ_{max} (ϵ (l^{-1} m^{-1})).
[a] G. O. Schenck and K. Gollnick, *Forsch. Landes Nordrhein-Westfallen*, Nr. 1256, Köln und Opladen, 1963.
[b] J. Paczkowski, J. J. M. Lamberts, B. Paczkowska and D. C. Neckers, *J. Free Radicals Biol. Med.*, *1* (1985) 341.
[c] S. M. Linden and D. C. Neckers, *Photochem. Photobiol.*, *47* (1988) 543.
[‡] The compound is not very soluble in CH_2Cl_2.
[d] J. J. M. Lamberts and D. C. Neckers, *Z. Naturforsch., Teil B*, *39* (1983) 474.
[e] D. Luttrull, O. Valdes-Aguilera, S. M. Linden, J. Paczkowski and D. C. Neckers, *Photochem. Photobiol.*, *47* (1988) 551.
[f] D. C. Neckers and J. Paczkowski, *Tetrahedron*, *42* (1986) 4671.
[g] 95% water/5% EtOH.
[h] DMF.
[i] Toluene.
[j] Cyclohexane.

TABLE 3
Emission properties of known derivatives of Rose Bengal



Compound		*Water*[a] (ϕ)	*MeOH*	*EtOH*	*MeTHF*	*Other*
R^1 = Na^+; R^2 = Na^+	Fl(R.T.)[b]	– (0.01)	573 (0.08)	566 (0.11)	587	*n*-PrOH (0.13)
	Fl(77 K)	–		569 620	–	*n*-BuOH (0.14)[c]
	Ph(77 K)[d]	–	702	717	723	
R^1 = Li^+; R^2 = Li^+;	Fl(R.T.)	–	572	–	–	
	Fl(77 K)	–		568 620	–	
	Ph(77 K)	–	–	716	–	
R^1 = K^+; R^2 = K^+	Fl(R.T.)	–	572	–	–	
	Fl(77 K)	–	–	569 620	–	
	Ph(77 K)	–	–	713	–	
R^1 = Cs^+; R^2 = Cs^+	Fl(R.T.)	–	573	–	–	
	Fl(77 K)	–	–	571 620	–	
	Ph(77 K)	–	–	712	–	
R^1 = TMA^+; R^2 = TMA^+	Fl(R.T.)	–	573	–	–	
	Fl(77 K)	–	–	589 610	–	
	Ph(77 K)	–	–	710	–	
R^1 = DEA^+; R^2 = DEA^+	Fl(R.T.)	–	573	–	–	
	Fl(77 K)	–	532	569 618	–	
	Ph(77 K)	–	–	713·15	–	
R^1 = TEA^+; R^2 = TEA^+	Fl(R.T.)	–	573	–	–	
	Fl(77 K)	–	530	569 617	–	
	Ph(77 K)	–	–	710·11	–	
R^1 = pip^+; R^2 = pip^+	Fl(R.T.)	–	571	–	–	
	Fl(77 K)	–	531	568 617	–	
	Ph(77 K)	–	–	708·10	–	
R^1 = CH_3; R^2 = Na^+	Fl(R.T.)	–	583·4	–	–	
	Fl(77 K)	–	–	571 620	–	
	Ph(77 K)	–	–	712·13	–	
R^1 = CH_3; R^2 = H^+	Fl(R.T.)	–	581	–	–	
	Fl(77 K)	–	535	571 624	–	
	Ph(77 K)	–	–	715	–	
R^1 = $CH_2\phi$; R^2 = Na^+	Fl(R.T.)	–	584·5	–	–	
	Fl(77 K)	–	–	573 625	–	
	Ph(77 K)	–	–	710	–	
R^1 = $CH_2\phi$; R^2 = H^+	Fl(R.T.)	–	581	–	–	
	Fl(77 K)	–	–	576 622·3	–	
	Ph(77 K)	–	535	695·6	–	

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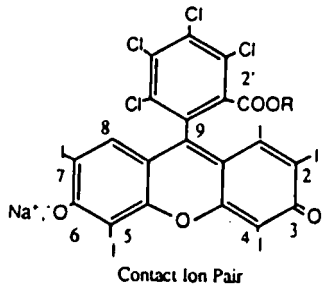
TABLE 3 *(continued)*

Compound		Water[a] (ϕ)	MeOH	EtOH	MeTHF	Other
R^1 = $CH_2ϕ$;	Fl(R.T.)	–	–	–	–	
R^2 = TMA$^+$	Fl(77 K)	–	– 533	576 618	–	
	Ph(77 K)	–	–	704	–	
R^1 = $CH_2ϕ$;	Fl(R.T.)	–	–	–	–	
R^2 = TEA$^+$	Fl(77 K)	–	– 534	575 618	–	
	Ph(77 K)	–	–	713	–	
R^1 = $CH_2ϕ$;	Fl(R.T.)	–	–	–	–	
R^2 = DEA$^+$	Fl(77 K)	–	– 533	576 618	–	
	Ph(77 K)	–	–	700-1	–	
R^1 = $CH_2ϕ$;	Fl(R.T.)	–	–	–	–	
R^2 = pip$^+$	Fl(77 K)	–	– 534-5	576 618	–	
	Ph(77 K)	–	–	702	–	
R^1 = ethyl;	Fl(R.T.)	–	585	590	–	
R^2 = TEA$^+$	Fl(77 K)	–	–	586 625	–	
	Ph(77 K)	–	–	710	–	
R^1 = ethyl;	Fl(R.T.)	–	592	600	–	
R^2 = Bu_4N^+	Fl(77 K)	–	–	586 623	–	
	Ph(77 K)	–	–	708	754	
R^1 = Z-Lys;	Fl(R.T.)	–	588	–	–	
R^2 = TEA$^+$	Fl(77 K)	–	– –	577 625	–	
	Ph(77 K)	–		719	–	
R^1 = Z-[Lys]-OMe;	Fl(R.T.)	–	588	–	–	
R^2 = TEA$^+$	Fl(77 K)	–	– –	580-1 625	–	
	Ph(77 K)	–	–	720-1	–	

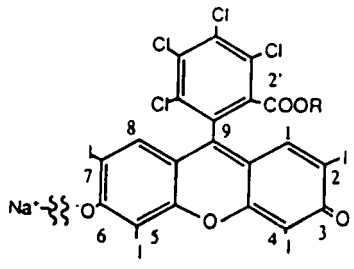
[a] All phosphorescence spectra were measured at 77 K unless otherwise indicated.
[b] $λ_{ex}$ = 500 nm unless otherwise noted.
[c] 20 °C.
[d] $λ_{ex}$ = 550 nm unless otherwise noted.

3.1. Substitution at C-6

Because of the sterically imposing *ortho* iodines at C-6, the organic reactivity of Rose Bengal, disodium salt, as a nucleophile is entirely at the C-2′ position and most of the known Rose Bengal derivatives which have been prepared take advantage of this reactivity. Unionized derivatives at C-6 show a typical spectrum regardless of whether the spectra are of covalent derivatives or of contact ions pairs.



Contact Ion Pair

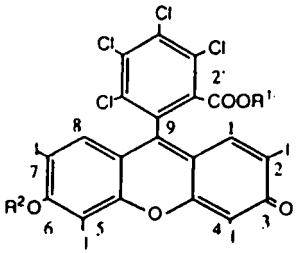


Solvent Separated Ion Pair

This typical spectrum is substantially blue shifted from the dye ionized at C-6 and has an order of magnitude lower ϵ at its two $λ_{max}$, 494 ± 3 nm and 404 ± 3 nm. The shorter wavelength $λ_{max}$ at 404 nm is particularly important because it distinguishes the unionized form of the dye from the spectrum of the dimer. Substituting a carbon group at C-6 requires special conditions and, once the substituted C-6 compound has been prepared, special care in handling. Aside from many C-6 protonated forms of C-2′ esters [11] and derivatives of the lactone of which the lactone diacetate is an example, there are only three known derivatives in which both C-2′ and C-6 are covalent [13]. To the best of our knowledge there are no cases of pure compounds having been isolated in which C-2′ is ionic and C-6 substituted. The one compound which has been reported, C-6-Me,C-2′ carboxylate has the right sort of spectrum ($λ_{max}$ (ϵ) (EtOH) = 493 nm (5850), 402 nm (6610); $λ_{max}$ (ϵ) (EtOH/acetone) = 486 nm (5430), 394 nm (5930)) [14] but it has not yet been isolated as a pure solid. The spectra of the three disubstituted derivatives which have been isolated are given in Table 4.

TABLE 4

Known derivatives of Rose Bengal functionalized at C-6 with a covalently bonded group



Compound	$CHCl_3$ $λ_{max}$ (ϵ)	Acetone/EtOH (1:1)
R^1 = $CH_2ϕ$;	497 (12800)	494 (21700)
R^2 = CH_3	403 (17900)	403 (29100)
R^1 = CH_3;	497 (11900)	492 (17100)
R^2 = CH_3	403 (16900)	402 (23700)
R^1 = $CH_2ϕ$;	497 (17400)	494 (15800)
R^2 = $(CH_3)_3Si$	403 (24100)	403 (22000)

To a greater or lesser degree the spectra of all C-6 ionic salts, whether they are substituted with a covalent group at C-2′ or not, are comprised of a mixture of the spectra of the C-6 ionic and the C-6 covalent form. Differences in the spectrum of one compound with another result from secondary effects on the structure of the bare anion such as hydrogen bonding and dimerization due to hydrophobic dye/dye molecular interactions. This is particularly obvious when the fluorescence spectra of the ammonium salts at room temperature and at reduced temperature are

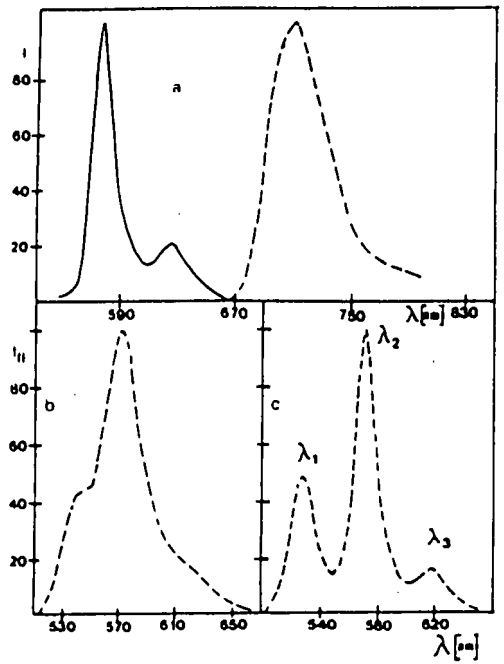


Fig. 2. Emission spectra of Rose Bengal derivatives. (a) Total emission spectra of Rose Bengal at 77 K. (b) Fluorescence emission of Rose Bengal, ditrimethylammonium salt in EtOH at ambient temperature. (c) Fluorescence emission of Rose Bengal, ditrimethylammonium salt in EtOH recorded at 77 K (λ_{ex} = 520 nm).

compared (Fig. 2). Thus the fluorescence spectrum at room temperature of the C-2',C-6 bistrimethylammonium salt shows a broad triad of peaks centered at 583 nm (Table 3). At reduced temperature the same compound shows three sharper peaks at 538 nm, 583 nm and 605 nm (Fig. 2). The shortest wavelength of these emissions is due to the contact ion pair whereas the higher wavelength pair of peaks is the result of the totally dissociated dianion [15].

Specific examples illustrate that similar principles affect the absorption spectra. For example the C-2' benzyl ester, C-6 protio form is red in MeOH and orange in methylene chloride. Thus it has the spectrum of the ionized form in the polar, protic solvent and of the contact ion pair or unionized form in the non-polar solvent methylene chloride [6, 16] (Fig. 3(a)). The tributylammonium salt, C-2' octyl ester is soluble in solvents ranging from ethanol/water to toluene (Fig. 3(b)). Its spectrum, even in an essentially non-ionizing solvent such as toluene, is that of the ionized xanthene [17]. The spectrum of the pyrilium salt is concentration dependent. In dilute solution the compound is totally ionized and is red, whereas in concentrated

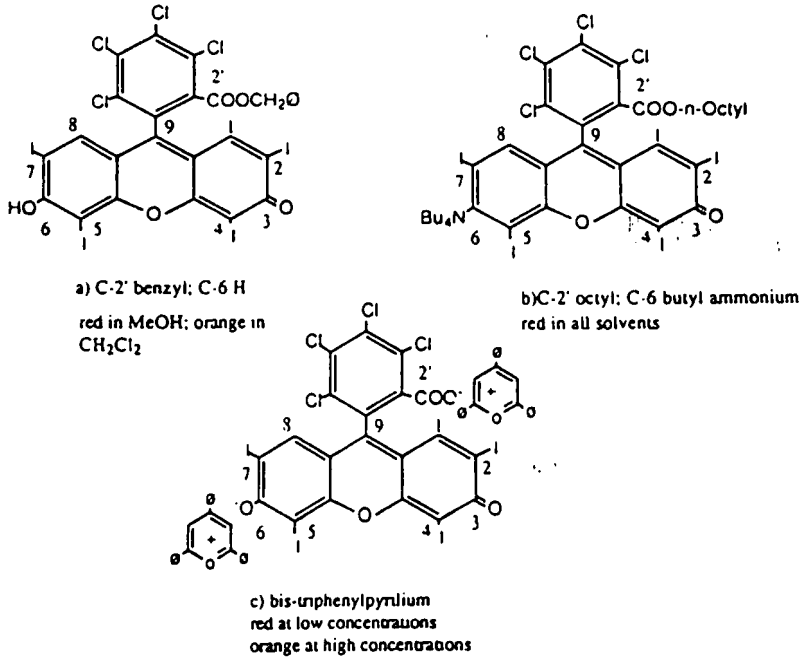


Fig. 3. (a) Rose Bengal, C-2' benzyl ester, C-6 H; (b) C-2' octyl ester; C-6 tetrabutylammonium salt; (c) C-2',C-6 bistriphenylpyrilium salt.

solution the compound is not fully ionized and the orange form predominates. The relative degree of ionization in dilute solution being highest is merely an example of the law of mass action (Fig. 3(c)).

3.2. C-2' substitution

In solvents in which the group at C-6 is totally ionized, C-2' substitution has a much smaller effect on the spectrum than C-6 substitution. A subtle 5 - 7 nm shift to the red is observed in the λ_{max} relative to the dianion in virtually every solvent in which both are soluble. This results from the inability of C-2' to neutralize partially the zwitterionic C-6$^-$,C-9$^+$ resonance contributor (Fig. 4). There is little difference in the extinction coefficients of Rose Bengal C-2' esters and the disodium salt.

3.3. Solvent effects

Solvent effects on the absorption spectra can be summarized as follows: if the compound is soluble in water, alcohols, and polar, non-protic solvents such as DMSO, DME and DMF, the λ_{max} increases in that order, *i.e.* it is most red shifted in polar, non-protic solvents. Compounds which are soluble in non-polar solvents such as CH_2Cl_2 are generally not soluble

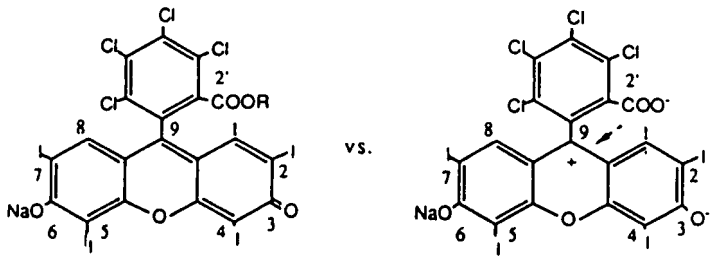


Fig. 4. C-2 esterification (left structure) prevents neutralization of the zwitterionic + charge at C-9. The C-2′ esters absorb at slightly longer wavelengths than Rose Bengal, C-2′,C-6 salts.

in water, and their absorption lies at about the same place in both alcohols and methylene chloride, but is shifted in polar, non-protic solvents to the red. λ_{max} also traces the hydrogen-bonding ability of the solvent — the shorter the wavelength of the maximum absorption, the stronger the hydrogen bond to the solvent from the xanthene. The same is true for the fluorescence maximum. All of these generalizations are made clear by a careful examination of the data in Tables 2 and 3. The absorption spectra

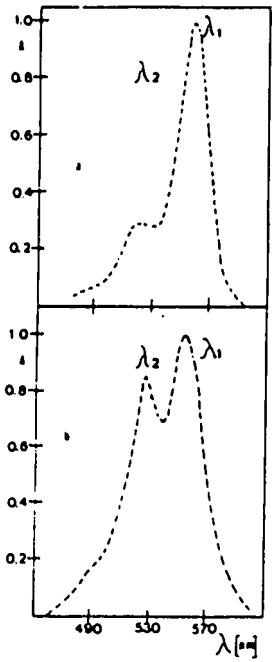


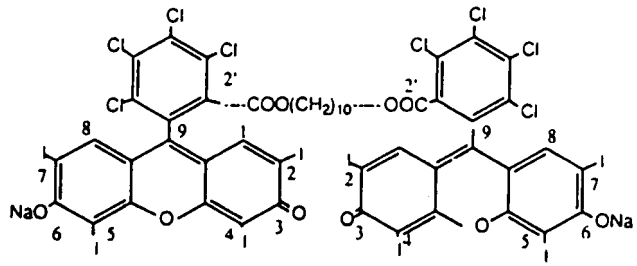
Fig. 5. Absorption spectra of (a) Rose Bengal in MeOH and (b) Rose Bengal, bis piperidinium salt, in CH_2Cl_2.

in methanol and methylene chloride (Fig. 5) indicates what solvation does to the appearance of the spectrum.

3.4. Aggregation effects

Rose Bengal, disodium salt, is soluble in water up to a concentration of approximately 10^{-3} M. The spectrum follows Beer's law only below 10^{-6} M whereupon subtle perturbations on the observed spectrum begin to appear. At higher concentrations the ratio of the two absorption maxima (λ_1/λ_2) decreases because of the formation of aggregates, the latter being driven by hydrophobic effects [18]. Rose Bengal dimer has the absorption spectrum in water shown in Fig. 6 [19]. In contrast to the monomer, the dimer absorbs at the shorter wavelengths but goes through a minimum at the longer wavelengths. Therefore as the concentration of dimer increases the absorption at 550+ nm decreases whereas the absorption at 525± nm remains the same or increases slightly. This accounts for the marked change in ratio of the longer-wavelength absorption to that at shorter wavelength ($\epsilon\lambda_1/\epsilon\lambda_2$) as a function of concentration in aqueous solution [20].

The effects of enforced aggregation have been studied in rationally synthesized dimers by Luttrull, Valdes-Aguilera, Linden, Paczkowski and Neckers [17]. The spectra of the Rose Bengal, C-2′ ethyl ester, C-6 sodium salt and the decamethylene dimer, bis sodium salt



bis Rose Bengal, C-2′ decamethylene, C-6 bis Na^+ salt.

are compared in Fig. 7. As is obvious from the comparison of the two spectra in Fig. 7, there is some broadening in the spectrum of the decamethylene dimer relative to the model. We have indicated that interaction between the two dye molecules leads to this perturbation in the normally sharp pair of peaks in the 525 - 555 nm region. This perturbation is a function of solvent and becomes particularly pronounced in water/ethanol of less than 20% EtOH where the very polar, protic solvent forces the non-polar organic Rose Bengals into a configuration which maximizes hydrophobic interactions. In comparing C-2′ decamethylene esters in which C-6 is differently substituted, as with either a sodium ion or an organic cation, when C-6 is Na^+ λ_{max} shifts to shorter wavelengths as the ethanol content is increased. When the cation at C-6 is organic, λ_{max} shifts to shorter wavelengths initially as the EtOH content of the solvent is decreased, but

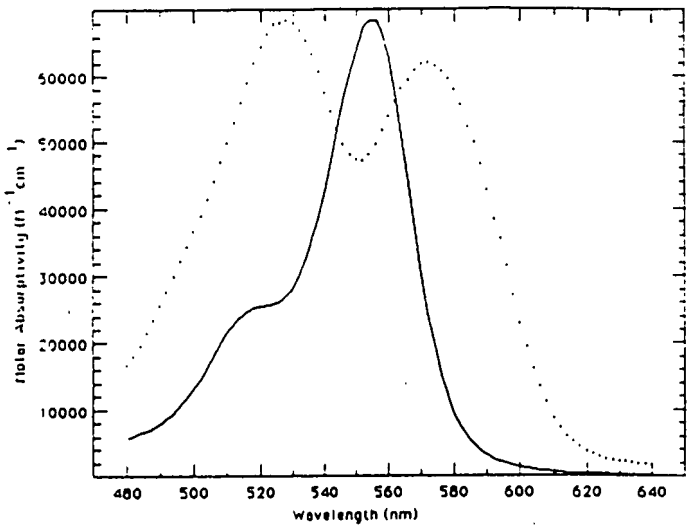


Fig. 6. Absorption spectrum of Rose Bengal, C-2′ ethyl ester 10^{-5} M in water (full line) and 10^{-5} M in 1.0 M KNO_3 (dotted line). The full line is the spectrum of the monomer; the dotted line is the spectrum of the dimer.

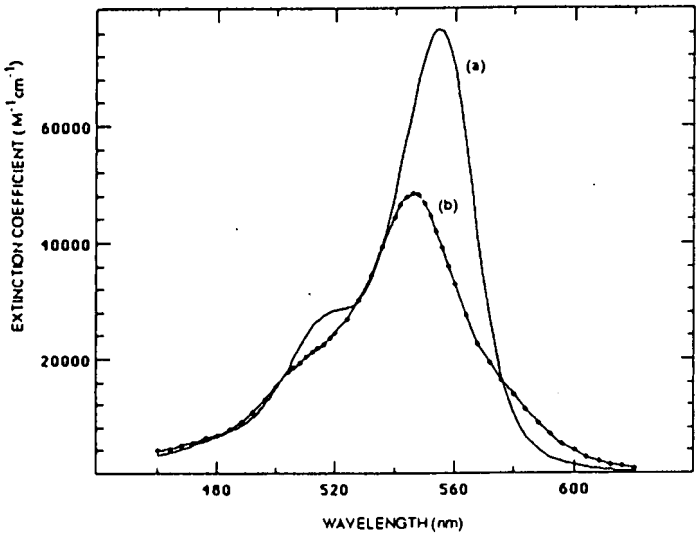


Fig. 7. Absorption spectra of (a) Rose Bengal, C-2′ ethyl ester and (b) C-2′-decamethylene, C-6 bis Na^+ salt.

to longer wavelengths when the EtOH content of the solvent is below 50%. The extinction coefficient also changes as the EtOH concentration of the solvent is decreased, decreasing after the EtOH is reduced below 20%.

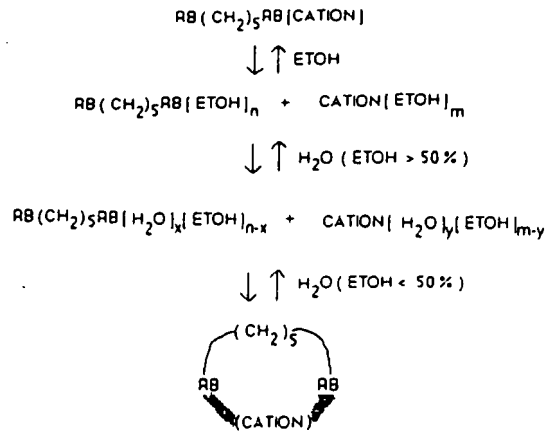
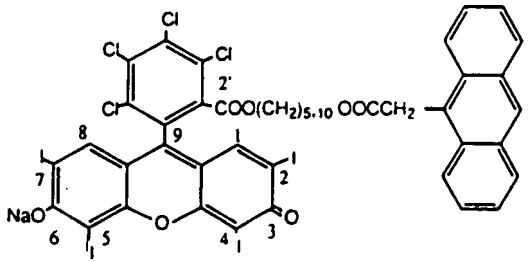


Fig. 8. Equilibria in ethanol for $RB(CH_2)_5RB$ complex formation.

The presence of intramolecular aggregates is confirmed by comparing the absorption spectra of compounds such as the bis C-2′ decamethyl ester and the excitation spectrum. We have proposed the equilibria outlined in Fig. 8 to explain these results.

The situation is similar with long-chain C-2′ anthracene esters of Rose Bengal



The absorption spectra of two such compounds are given in Fig. 9. In the case with five methylene groups (I) there is perturbation in the Rose Bengal spectrum which is caused by the anthracene moiety positioned five carbons away. This perturbation on the Rose Bengal spectrum is more significant in the case of the decamethylene system (II). Based on the lack of emission from the aggregated form as shown by comparing the excitation and emission spectra, an equilibrium constant for the intramolecular complex [21] is calculated as 1.78 for the C-5 compound and 10.06 for the C-10. The triplet lifetime of only the decamethylene derivative (II) is lower than expected (τ = 2.90 μs *vs.* 8.0 μs for Rose Bengal, C-2′ ethyl ester, C-6 Na^+ salt), though there is no evidence either of anthracene triplet being formed or of electron transfer products such as Rose Bengal radical anion.

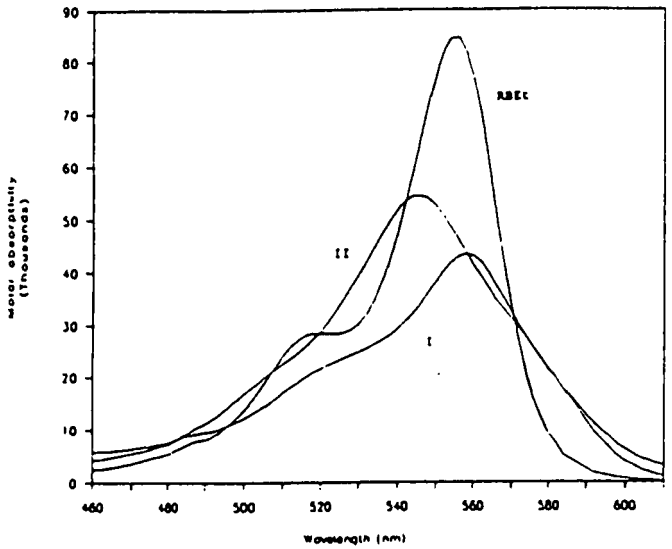


Fig. 9. Absorption spectra of Rose Bengal, C-2′ pentamethylene anthranoate (I) and Rose Bengal, C-2′ decamethylene anthranoate (II) compared with Rose Bengal, C-2′ ethyl ester (RBEt).

In polymeric systems the effects of proximity are threefold: effects on the absorption and emission spectra, effects on intermolecular energy transfer as measured by singlet oxygen yields, and intramolecular self-quenching. Spectral effects have been studied in two cases. In the first, poly(styrene-co-chloromethylvinylbenzenes) were treated with greater and greater concentrations of Rose Bengal in DMF [22]. In the second case, anionically polymerized styrenes were terminated with dibromoethane and the resulting CH_2Br end-capped polymer treated with Rose Bengal, producing linear polystyrene end-capped with Rose Bengal esters [23].

The effect of increasing the loading of Rose Bengal on a poly(styrene-co-chloromethylstyrene) on the absorption spectrum is, even in non-polar solvents such as methylene chloride, to produce results which can be attributed to aggregation. The absorption data for five poly(styrene-co-chloromethylstyrenes) differently loaded with increasing mg of Rose Bengal per gram of polymer are shown in Table 5.

The effects of loading on the absorption spectra in CH_2Cl_2 solution and as a cast film are in the same direction but of different magnitude. We have interpreted this as meaning that the Rose Bengal moieties in the film are surrounded by the non-polar polymer whereas in CH_2Cl_2 solvation effects predominate. There is a limited red shift as a function of dye concentration on the polymer backbone, but a major change in the relative extinction coefficients at the longer and shorter wavelengths. There is also

TABLE 5

Absorption spectra of poly-RBs in CH_2Cl_2 and in polymer films

Polymer[a]	*CH_2Cl_2 solution*			*Thin films*		
	λ_1(max)	λ_2(max)	$\epsilon_{\lambda 1}/\epsilon_{\lambda 2}$	λ_1(max)	λ_2(max)	$\epsilon_{\lambda 1}/\epsilon_{\lambda 2}$
poly-RB-51	571.0	523.0	3.16	576.5	534.5	2.19
poly-RB-102	572.0	532.0	2.68	578.0	537.0	1.83
poly-RB-152	572.0	532.0	2.42	578.0	537.0	1.79
poly-RB-450	572.5	532.0	1.94	577.5	537.0	1.72
poly-RB-610	572.5	532.0	1.59	578.0	536.5	1.63

[a]mg Rose Bengal per gram of poly(styrene-co-chloromethylstyrene;3:1).

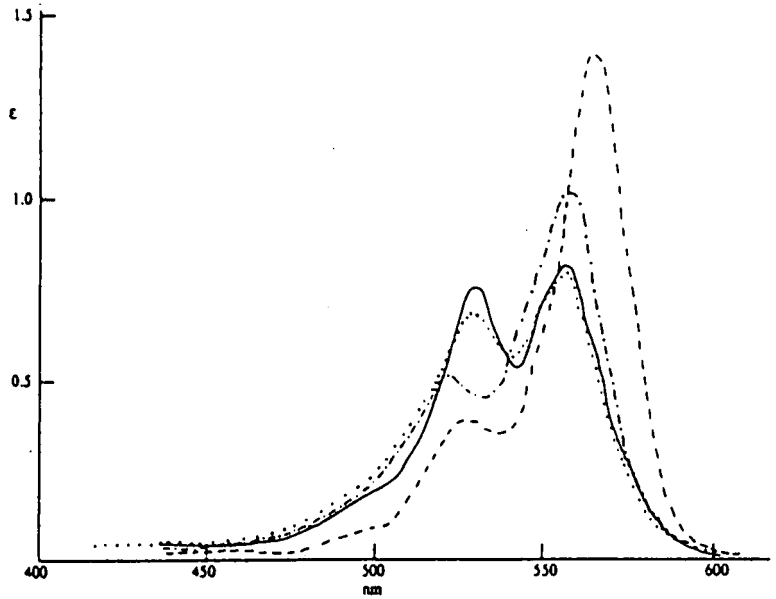


Fig. 10. Absorption spectra of polystyrene end-capped with Rose Bengal.

	$\lambda_{max\,1}$	$\lambda_{max\,2}$	$\epsilon_{\lambda 1}$ (g^{-1} cm^{-1} l^{-1})	$\epsilon_{\lambda 2}$ (g^{-1} cm^{-1} l^{-1})	$\epsilon_{\lambda 1}/\epsilon_{\lambda 2}$
– – – DMF	572	529	1.628	0.470	3.46
– · – CH_2Cl_2	562	523	1.167	0.587	1.99
····· Toluene	562	534	0.935	0.780	1.20
—— Cyclohexane	562	534	0.932	0.870	1.07

an obvious broadening in the spectrum particularly at shorter wavelengths. We attribute these spectral changes to enforced aggregation of neighbor dyes on the polymer backbone. Unfortunately at the time these polymers were synthesized the fluorescence spectra were recorded only in thin films, and they were not accompanied by excitation spectra. The changes in spectra in the film can be attributed to self-absorption effects.

Rose Bengal end-capped polystyrenes show a marked change in spectrum as a function of solvent (Fig. 10) owing to differential solvating ability at C-6 in solvents of differing polarity. Though the polymers in Fig. 10 are identical, significant shifts in the spectra are observed from solvent to solvent.

Most interesting spectral changes occur with these polymers when they are irradiated in hydrocarbon solvents such as cyclohexane and toluene. When irradiated in these solvents the spectra of the polymers change in the direction of increased aggregation (Fig. 11). We have suggested that when irradiated the terminae of the polymers interact with the resulting product, which is dimeric at the ends rather than monomeric. Perhaps the driving force for this conformational change is electron transfer from one Rose Bengal terminus to another, followed by back electron transfer (Fig. 12).

Transient absorption spectra have been reported for certain intramolecular energy transfer events particularly in the case of anthracene derivatives of Rose Bengal [24], though their interpretation must be made with extreme caution. In MeOH, Rose Bengal C-2′ ethyl ester undergoes bimolecular self-quenching with the appearance of three main absorption bands at 380, 465 and 605 nm 0.3 μs after the flash. These bands decay

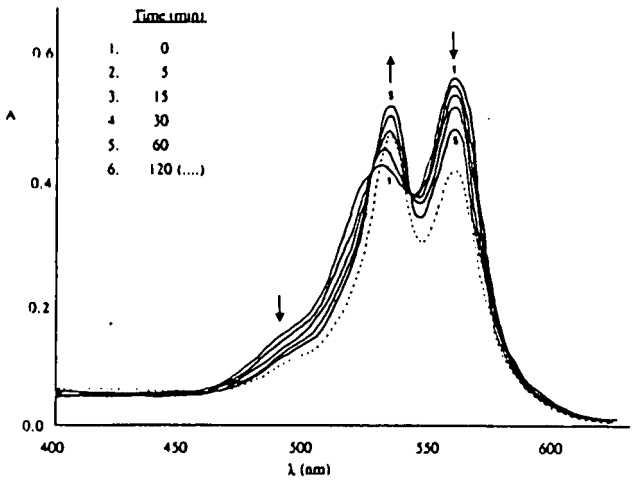


Fig. 11. Change in spectrum of polystyrene end-capped with Rose Bengal upon irradiation.

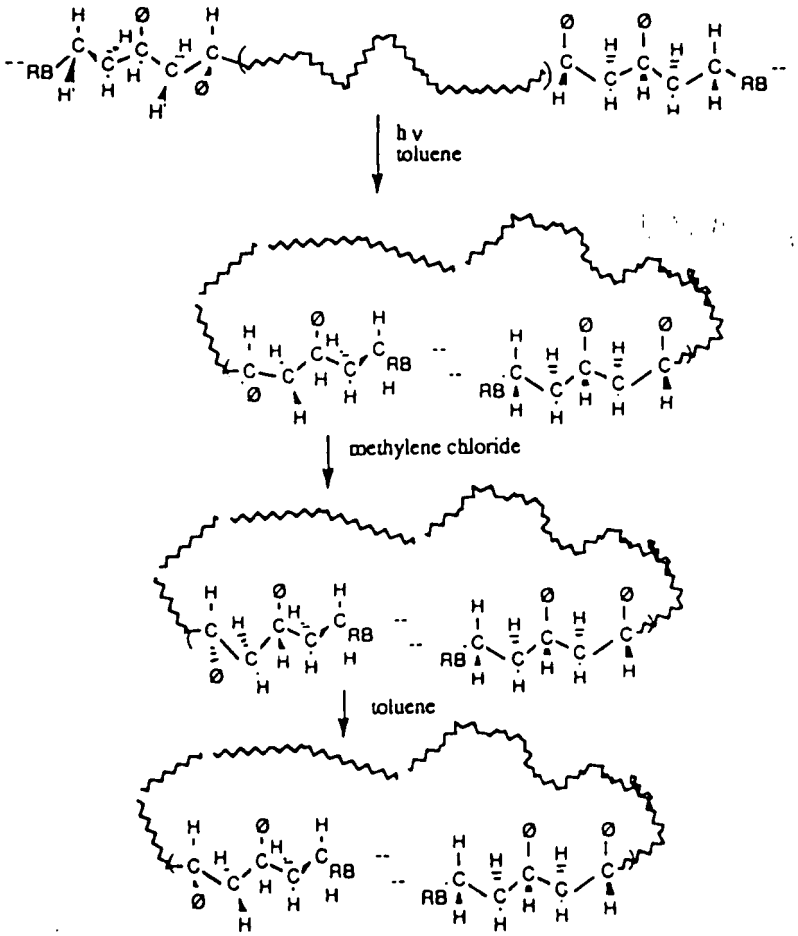


Fig. 12. Photochemically driven reorganization in polystyrenes end-capped with Rose Bengal.

in several hundred microseconds to bands at ≈ 420 nm and ≈ 450 nm respectively. Analysis of these transients shows that the triplet absorbs at > 600 nm with $\tau_T \approx 16$ μs. This triplet lifetime is strongly dependent of RBEt concentration in the concentration range $(1 - 10) \times 10^{-5}$ M with a self-quenching rate constant of 7.8×10^7 M^{-1} s^{-1}. At shorter wavelengths the spectra are complicated by more than one species absorbing, but the peak at 420 nm grows in at a rate which is the same as the rate by which the triplet at ≈ 600 nm is being self-quenched. We suggest that this species is the radical anion. The radical cation grows in also at 450 nm [25].

4. Quenching of Rose Bengal singlets and triplets: oxygen

The triplet of Rose Bengal, disodium salt, is "completely quenched" by oxygen in polar solvents producing singlet oxygen and superoxide radical ion. Discovered originally by Jodlbauer and von Tappeiner [26], it is this property more than any other which was responsible for the widescale use of Rose Bengal in the first place. Rate constants for oxygen quenching are at the diffusion-controlled limit under conditions of air saturation in all solvents. The details of this energy transfer have been described elsewhere but the quantum yields of singlet oxygen formation, fluorescence and intersystem crossing in MeOH at 20 °C are compared for four of the xanthenes in Table 6.

TABLE 6

Quantum yield of fluorescence, triplet formation and singlet oxygen formation for simple xanthenes[a]

Compound	ϕ_f	ϕ_t	$\phi(O_2)$[b]
Rose Bengal, dianion	0.08	0.76	0.76
Erythrosin, dianion	0.08	0.62	0.62
Eosin, dianion	0.63	0.28	0.39
Fluorescein, dianion	0.93	0.03	0.09

[a] Data from Gollnick and Schenck [8]. Singlet oxygen reactions measured at [dye] = 3.0×10^{-4} using 2,5-dimethylfuran as the singlet oxygen trap; solvent MeOH at 20 °C.
[b] Quantum yields are a function of solvent, and are also likely a function of dye concentration.

Singlet lifetimes, fluorescence quantum yields and singlet oxygen quantum yields are compared for Rose Bengal, Erythrosin, Eosin and fluorescein in a number of different solvents in Table 7. In virtually every case water differs from the other solvents studied. Reference is made to Table 1 for additional data on Rose Bengal in water.

Singlet oxygen yields for several monomeric derivatives of Rose Bengal have been measured by Linden and Neckers. Because Rose Bengal, disodium salt, is only soluble in polar solvents certain of the oxidation products formed in singlet oxygen reactions may be consumed by these solvents in secondary reactions. Classic examples include 2,5-dimethylfuran [27] which produces the hydroperoxide hemiacetal rather than the subsequently isolated intermediate cyclobutadiene ozonide [28]. Singlet oxygen quantum yields from those Rose Bengal derivatives which have been reported are collected in Table 8 [29]. In polar solvents the values for each of the compounds do not vary much. In non-polar solvents, however, other processes which will be discussed later come into play.

Superoxide radical anion ($O_2^{\cdot-}$) was first reported formed from Rose Bengal by Srinivasan *et al.* [30]. In a clever experiment $O_2^{\cdot-}$ was detected

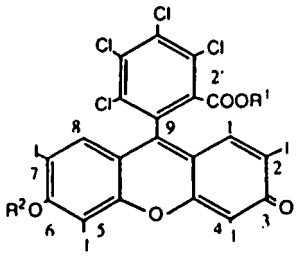
TABLE 7

Photophysical properties of simple xanthenes [8]; singlet lifetime as a function of solvent $\tau \times 10^9$ {ϕ_f}{γO_2}

Compound	*n-BuOH*	*n-PrOH*	*EtOH*	*MeOH*	*Water*
Rose Bengal dianion	0.77[0.14]{0.73}	0.71[0.13]{0.74}	0.69[0.11]{0.78}	0.44[0.08]{0.76}	0.05[0.01]
Erythrosin dianion	0.55[0.10]{0.65}	0.55[0.10]{0.65}	0.55[0.10]{0.65}	0.44[0.08]{0.62}	0.11[0.02]
Eosin dianion	3.15[0.68]{0.39}	3.21[0.71]{0.40}	3.34[0.72]{0.43}	2.92[0.63]{0.39}	0.51[0.11]
Fluorescein dianion	–	–	4.48[1.0]{0.07}	4.16[0.93]{0.09}	3.72[0.83]

TABLE 8

Singlet oxygen yields from derivatives of Rose Bengal [1, 2]



R^1; R^2	ϕO_2 [a] (MeOH)	CH_2Cl_2
ϕ_2I^+; ϕ_2I^+;	0.66	[b]
ϕCH_2; ϕ_2I^+;	0.74	[b]
ϕCH_2; $\phi_3PCH_2\phi$	0.76	—
ϕCH_2; triphenylpyrilium	0.70	[b]
ϕCH_2; CH_3SO_2	0.73	—
$\phi_3PCH_2\phi$; $\phi_3PCH_2\phi$	0.74	—
Et; H	0.73	0.61
TEA; TEA	0.72	0.48
ϕCH_2; TEA	0.74	0.67
Et; TEA	0.74	0.71
Et; OAc	—	0.61
$(CH_2)_7CH_3$; TBA	—	0.40 (toluene)

[a] Data from S. M. Linden and D. C. Neckers, *Photochem. Photobiol.*, *47* (1988) 543.
[b] In CH_2Cl_2 rapid bleaching via oxidative electron transfer precludes energy transfer. TEA = triethylammonium; TBA = tributylammonium.
[c] Solvent in all cases MeOH; singlet oxygen trap is 2,3-diphenyl *p*-dioxene.

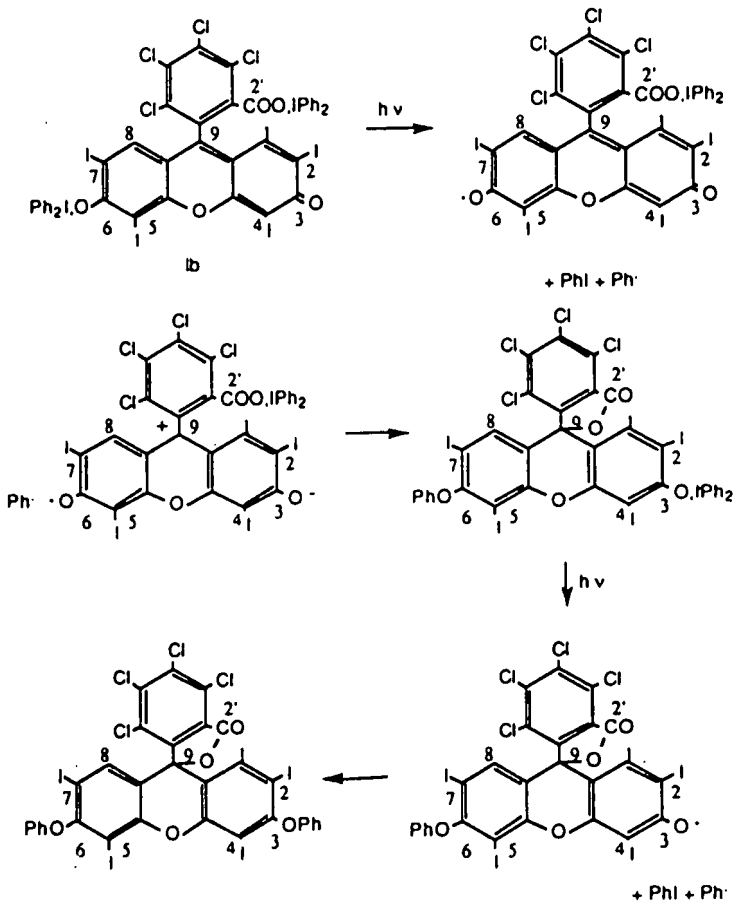
with superoxide dismutase and an oxygen electrode in water in the presence of sulfite. Rodgers and Lee later confirmed this result quantitatively [31] in water where 25% superoxide was observed. Superoxide formation may require something other than direct electron transfer from Rose Bengal triplet. The energetics allow self-quenching electron transfer followed by electron transfer from the radical anion of Rose Bengal to dioxygen. The mechanism, however, is bimolecular in Rose Bengal and suggests that the O_2^- yield should increase with the concentration of the dye. Though there are hints in the literature that this is the case [32], complete quantitative data on the yield of superoxide as a function of Rose Bengal concentration have not yet been reported [33].

5. Radical formation–dye oxidation and dye reduction

Eaton, in a review, reports that many xanthene dye reducing agents have been used as initiators of vinyl polymerization. Presumably these

are triplet reactions [34]. The oxidation and reduction potentials of those Rose Bengals which have been reported are given in Table 9.

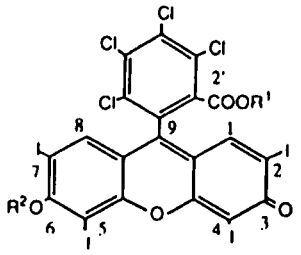
In non-polar solvents the iodonium salts, whether esterified at C-2′ or not, bleach extremely rapidly. In each of these cases the gegen ion, ϕ_2I^+, is easily reduced (ϵ_{red} = 0.50 eV) by the excited singlet state of the dye. Therefore the bleaching occurs by oxidative electron transfer from the dye to the gegen ion. Products deriving from this oxidation have been isolated and are shown below.



Methylene chloride solutions of the iodonium salts of Rose Bengal bleach from the singlet state in a few seconds in room light. The resulting phenyl radicals have been reported to initiate acrylate polymerization [35]. Other onium salts of Rose Bengal (Tables 2 and 3) bleach much more slowly and do so by a reductive mechanism.

TABLE 9

Oxidation and reduction of derivatives of Rose Bengal [34]



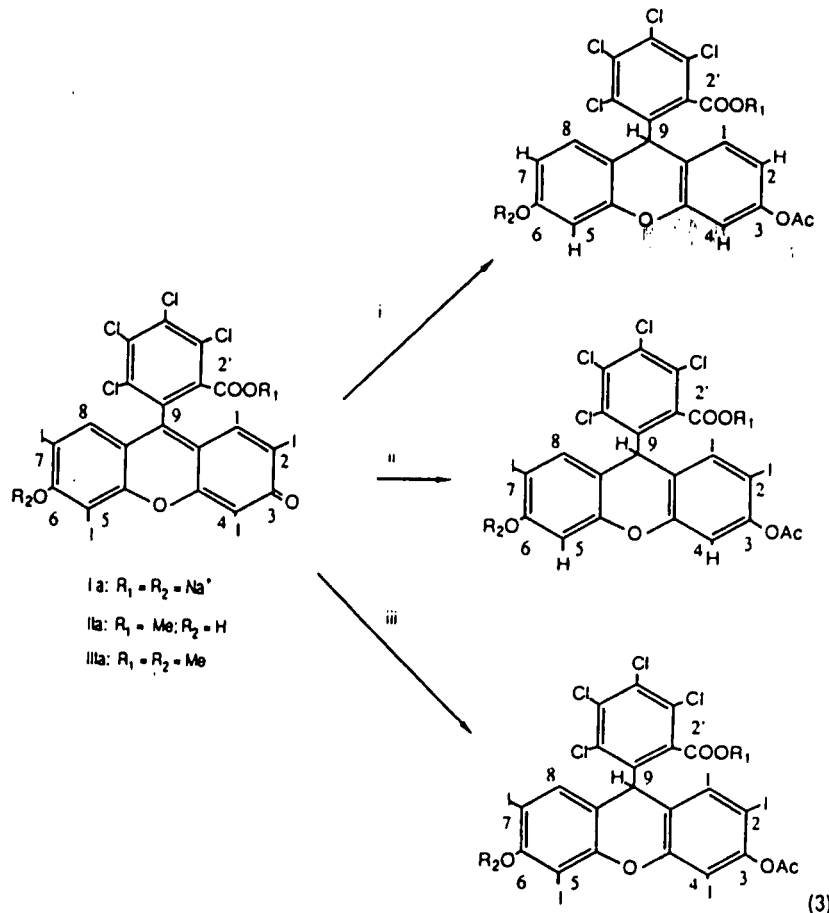
Compound: R^1; R^2	E_{ox} [a] (eV)	E_{red} (eV)
Na^+; Na^+	0.86ev	−1.03ev
ϕ_2I^+; ϕ_2I^+	0.86	−1.00
$\phi_3PCH_2\phi$; $\phi_3PCH_2\phi$	0.85	−1.05
Bu_4N^+; Bu_4N^+	0.90	−1.01
$CH_2\phi$; Na^+	0.92	−0.905
$CH_2\phi$; ϕ_2I^+	0.95	−0.895
ϕCH_2; $\phi_3PCH_2\phi$	0.93	−0.875
$CH_2\phi$; $CH_3SO_2^+$	0.94	−0.865
CH_3; Na^+	0.98	−0.86

[a] See notes in ref. 34 for experimental details.

The products of leuco Rose Bengal prepared under reducing conditions have been isolated by Zakrezewski [36]. Though reported by earlier workers to produce tetrachlorofluorescein [37] the primary photoreduction product of Rose Bengal is the dihydro compound (Fig. 13).

6. Conclusion

This review covers Rose Bengal chemistry, spectroscopy, photochemistry and photophysics. Because Rose Bengal has so many applications in biological and health sciences, a subsequent review will discuss the many and varied applications of Rose Bengal and its derivatives. Because Rose Bengal has been of specific interest to the author and his students, no attempt has been made to discuss the other xanthenes. In particular Eosin and fluorescein are extremely important dyes as well. These will also be the subject of a future review [38].



(3)

Fig. 13. Photoreduction of Rose Bengal under the following conditions. (i) (1) Zn, HOAc 24 h reflux; (2) Ac_2O, HOAc. (ii) (1) Zn, HOAc, 60 °C, 24 h; (2) Ac_2O, HOAc. (iii) $(HOCH_2H_4)_3N$, DME, 4 h, $h\nu$ [35]; (2) Ac_2O.

Acknowledgment

This work has been supported by the National Science Foundation (Division of Materials Research and Chemistry Division) and the National Institutes of Health (National Cancer Institute). The author is extremely grateful for the continued support of these agencies. The author also recognizes the outstanding contributions of the many students and postdoctoral associates who have worked in his laboratories on this project over the years. Each of these individuals is named in the references to this review.

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3 Bengalis was a widely recognized pattern used for cotton. Gnehm's archives, ETH Library, Zürich.

4 Only Bengali women wear the red spot at the center of the forehead. The decorative spot worn in the middle of the forehead by other Indian women is, today, pasted on.

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38 The author would be pleased to receive from readers any and all information about any of the xanthenes, published or unpublished.

Exhibit M

ALL-STATE® LEGAL 800-222-0510 EDS11 RECYCLED

US005780052A

United States Patent [19]

Khaw et al.

[11] Patent Number: 5,780,052

[45] Date of Patent: Jul. 14, 1998

[54] COMPOSITIONS AND METHODS USEFUL FOR INHIBITING CELL DEATH AND FOR DELIVERING AN AGENT INTO A CELL

[75] Inventors: **Ban An Khaw**, Milton; **Vladmir P. Torchilin**, Charlestown; **Jagat Narula**; **Imran Vural**, both of Brookline, all of Mass.

[73] Assignee: **Northeastern University**, Boston, Mass.

[21] Appl. No.: **427,676**

[22] Filed: **Apr. 24, 1995**

[51] Int. Cl.[6] **A61K 9/127**

[52] U.S. Cl. **424/450**; 436/829

[58] **Field of Search** 424/450; 436/829

[56] **References Cited**

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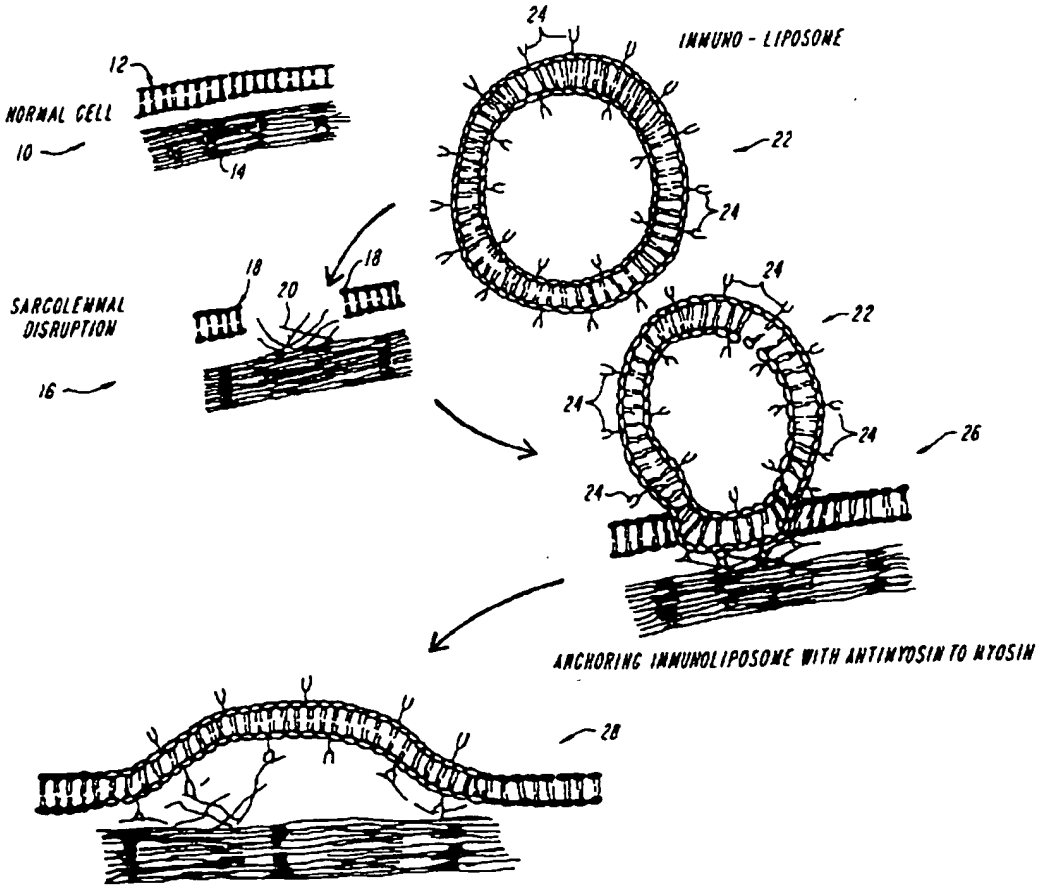
Primary Examiner—Gollamudi S. Kishore

Attorney, Agent, or Firm—Weingarten, Schurgin, Gagnebin & Hayes LLP

[57] **ABSTRACT**

The invention relates to methods of salvaging a target cell from cell death, comprising contacting a target cell having a disrupted cell membrane with a specific affinity reagent-liposome conjugate in an amount effective and for a time sufficient to allow the conjugate to prevent cell death due to membrane disruption; and determining the viability of the target cell. Methods of delivering a selected agent into a damaged target cell for diagnosis and therapy are also disclosed.

3 Claims, 8 Drawing Sheets
(9 of 12 Drawing(s) in Color)



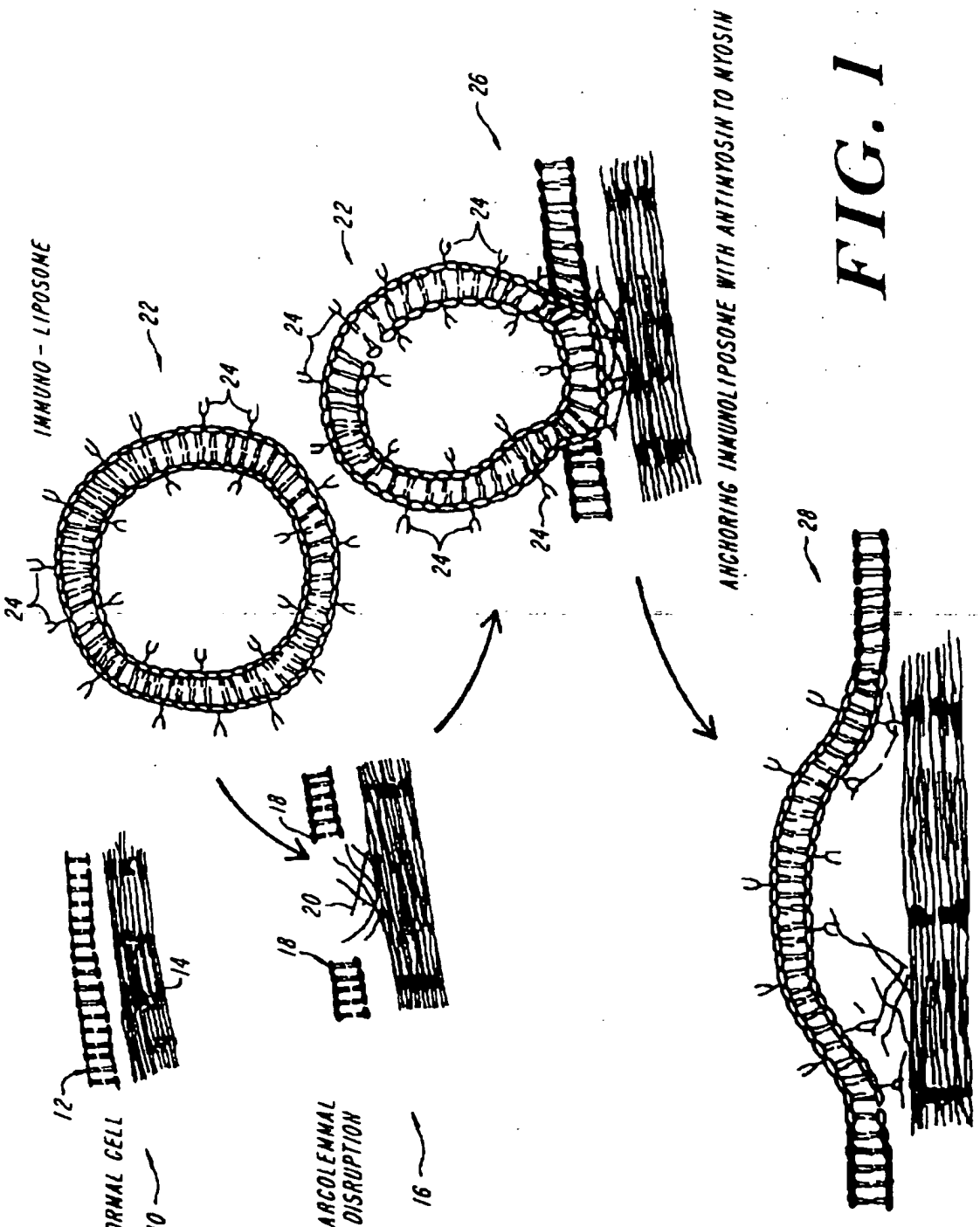

IMMUNO - LIPOSOME
22
24
26
NORMAL CELL
10
12
14
SARCOLEMMAL DISRUPTION
16
18
20
ANCHORING IMMUNOLIPOSOME WITH ANTIMYOSIN TO MYOSIN

FIG. 1

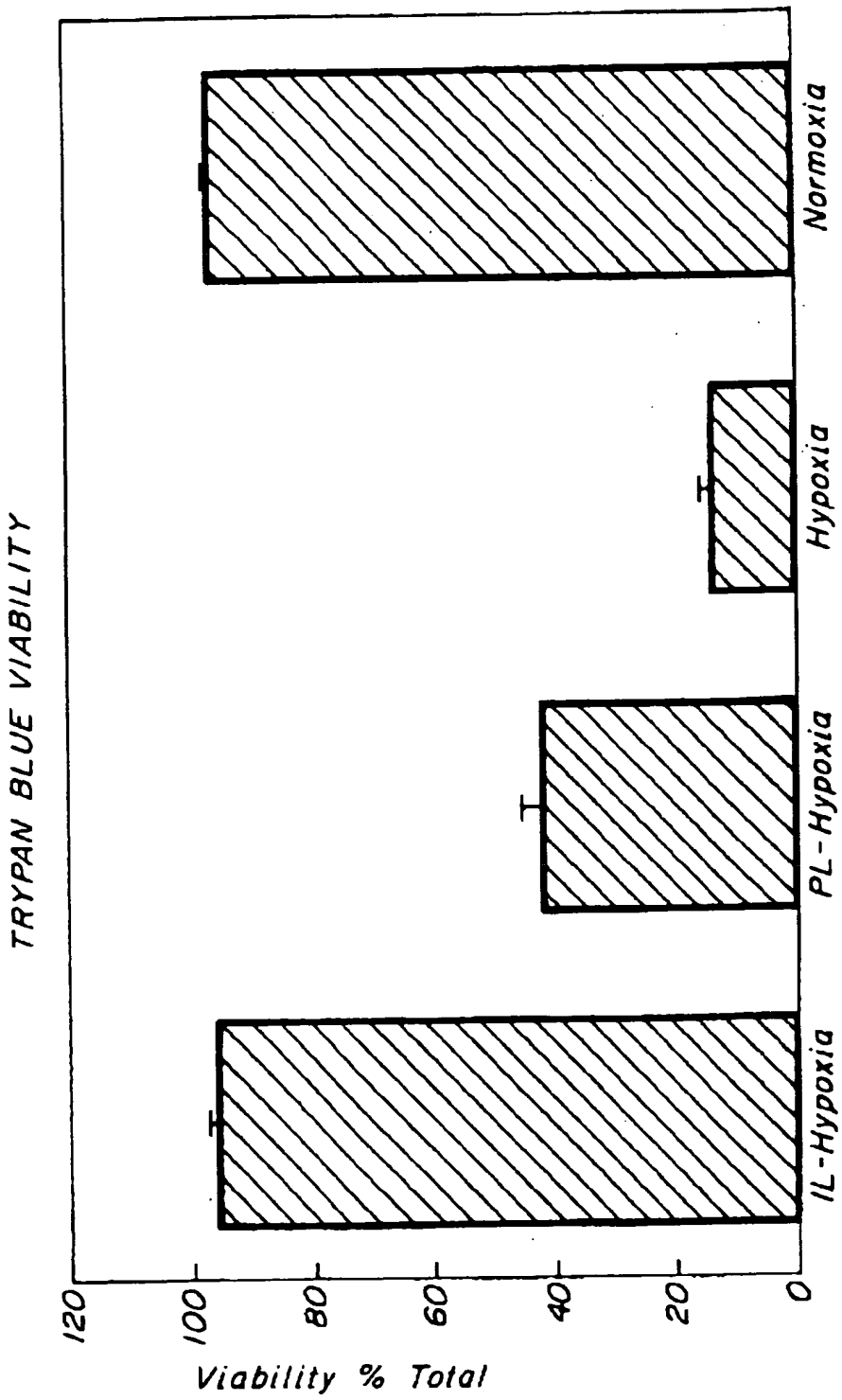

TRYPAN BLUE VIABILITY
Viability % Total
120
100
80
60
40
20
0
IL-Hypoxia
PL-Hypoxia
Hypoxia
Normoxia
mean

FIG. 2

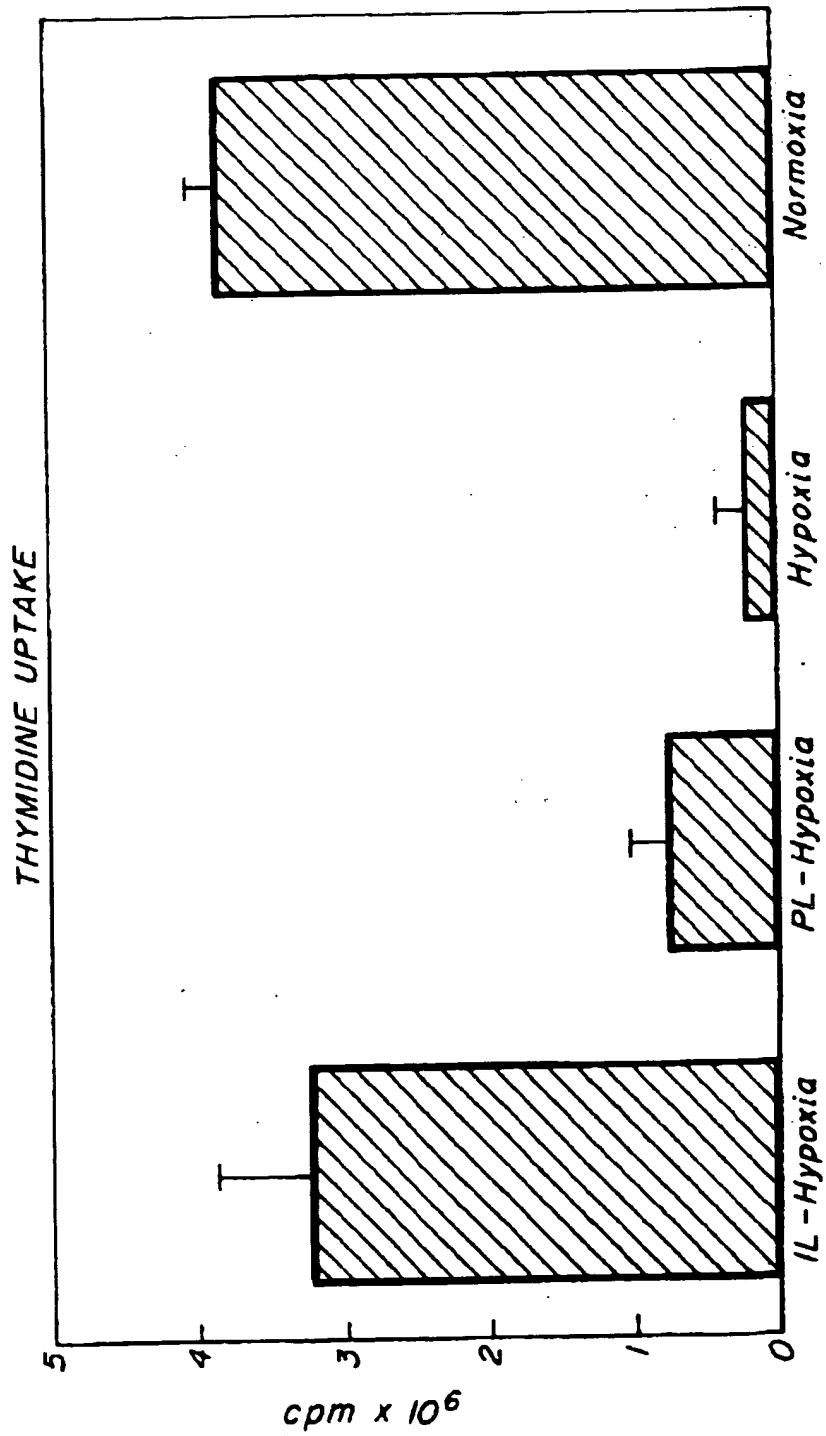

THYMIDINE UPTAKE
cpm x 10^6
0
1
2
3
4
5
Normoxia
Hypoxia
PL - Hypoxia
IL - Hypoxia
mean

FIG. 3



FIG. 4A

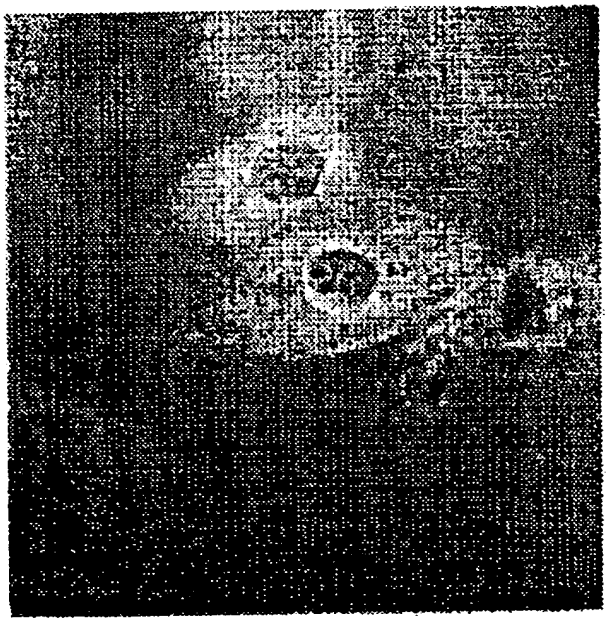


FIG. 4B

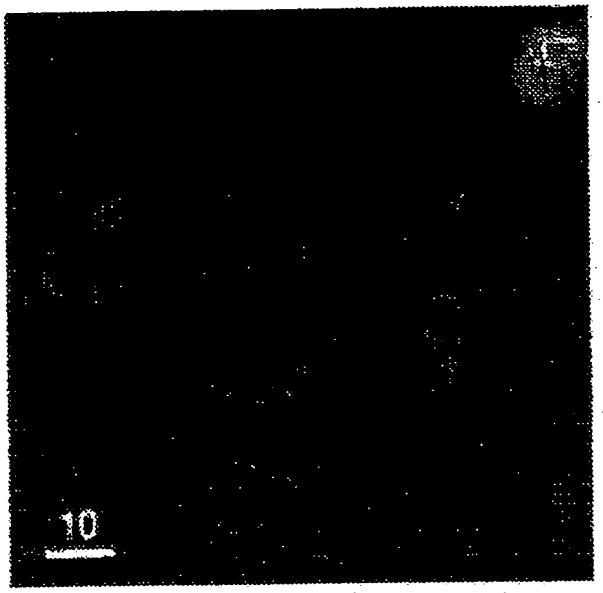


FIG. 5A

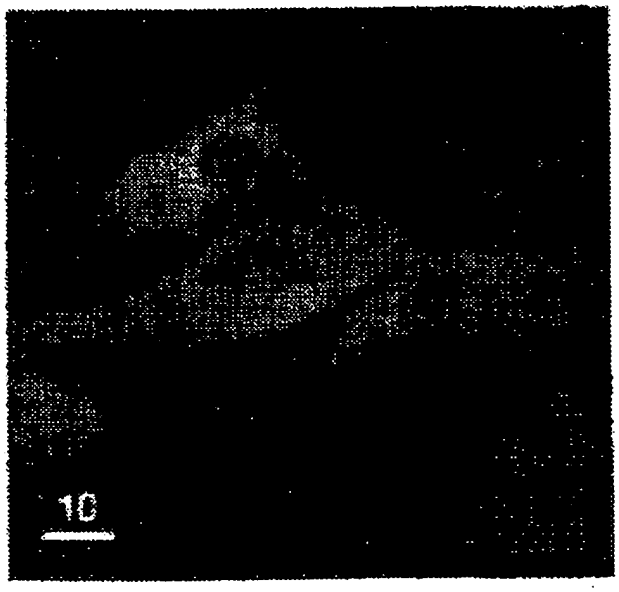


FIG. 5B



FIG. 6A

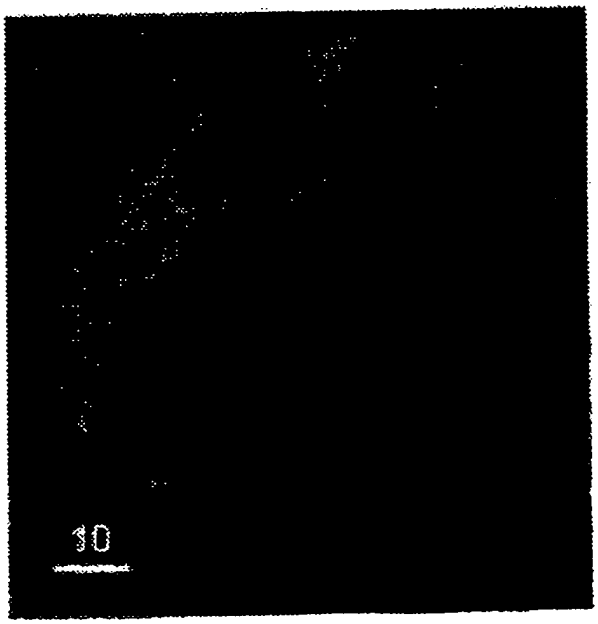


FIG. 6B

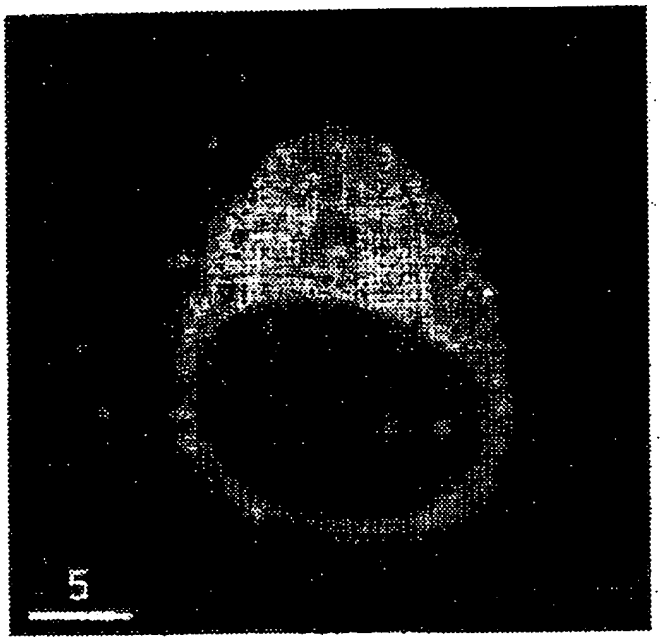


FIG. 7A



FIG. 7B

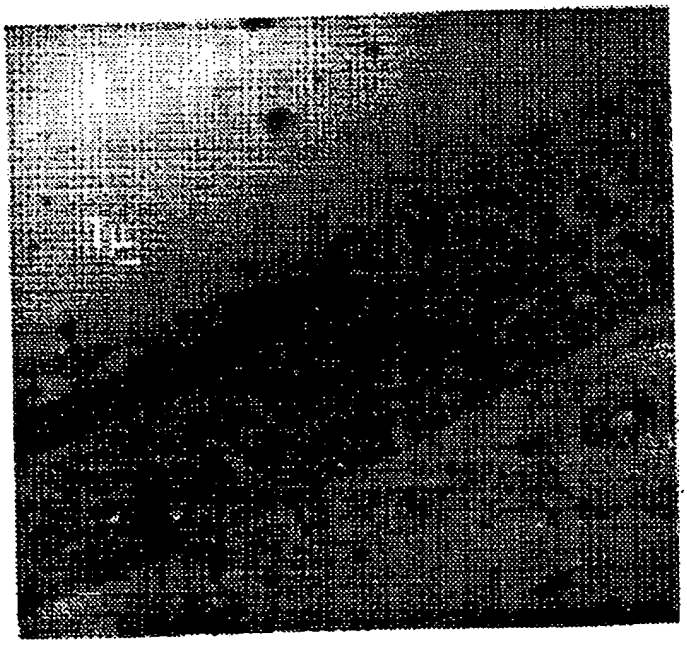


FIG. 8

COMPOSITIONS AND METHODS USEFUL FOR INHIBITING CELL DEATH AND FOR DELIVERING AN AGENT INTO A CELL

FIELD OF THE INVENTION

The invention relates to compositions and methods use inhibiting cell death and for delivering an agent into a cell.

BACKGROUND OF THE INVENTION

Proteins and phospholipids are the most abundant constituents of cellular membranes. a phospholipid molecule having a polar head group and two nonpolar. hydrophobic fatty acyl chains. Phospholipids can exist in different forms in aqueous solutions. among which are spherical phospholipid bilayer structures or liposomes. which have an aqueous interior.

Liposomes have been shown to be useful as drug delivery vehicles. A number of reviews have described studies of liposome production methodology and properties. their use as carriers for drugs and their interaction with a variety of cell types. See. for example. "Liposomes as Drug Carriers." Wiley and Sons. N.Y. (1988). and "Liposomes from Biophysics to Therapeutics." Marcel Dekker. N.Y. (1987).

Delivery of drugs using liposomes allows for noninvasive treatment of diseases. Targeting of an organ or tissue type may be made more efficient using immunoliposomes. i.e.. liposomes which are conjugated to an antibody specific for an organ-specific or tissue-specific antigen. Thus. one approach to targeted drug delivery is the use of drug-laden liposomes that have been made target-specific by incorporation of specific antibodies on the liposome surface. Such a system has been described using antimyosin antibody for targeting of liposomes to infarcted myocardium (Torchilin et al.. Biochem. Biophys. Res. Comm. 89:1114. 1979. hereby incorporated by reference). Normal myocardial cells with intact cell membranes do not permit extracellular macromolecules. such as antimyosin antibody. to traverse the cell membrane and interact with the intracellular constituents. However. necrotic myocytes with membrane disruptions can no longer keep antimyosin antibody from interacting with intracellular myosin exposed to the exterior through cell membrane breaches (Khaw et al.. Hybridoma 3:11–23. 1984). Immunoliposome-associated reagents have been reported to result in less than optimal accumulation at target sites. possible due to sequestration by the reticuloendothelial system. primarily by the liver and spleen. Torchilin et al. (FASEB Journal 6:2716. 1992) report on enhancement of circulation times using polyethylene glycol-coated immunoliposomes.

SUMMARY OF THE INVENTION

The invention is based on the discovery that specific affinity reagent-liposome conjugates may be contacted with cells that are in the process of dying so as to inhibit cell death. The invention encompasses two aspects. i.e.. inhibition of cell death via cell membrane sealing. and delivery of an agent to a cell via cell membrane sealing. Both aspects of the invention utilize specific affinity reagent-liposome conjugates.

Thus. one aspect of the invention is directed to a method of salvaging a target cell from cell death. comprising contacting a target cell having a disrupted cell membrane with a specific affinity reagent-liposome conjugate in an amount effective and for a time sufficient to allow the conjugate to prevent cell death due to membrane disruption; and determining the viability of the target cell.

This aspect of the invention also involves contacting a specific affinity reagent-liposome conjugate. in which the specific affinity reagent portion recognizes and binds to an intracellular antigen. with a target cell having a disrupted cell membrane so as to allow the specific affinity reagent portion to adhere to the intracellular antigen. the adherence resulting in initially plugging the lesions in the membrane followed by sealing of the cell membrane and inhibiting or arresting the process of necrosis or cell death.

In a second aspect. the invention is directed to contacting a specific affinity reagent-liposome conjugate with a target cell to which an agent is to be delivered. the agent being carried in the liposome portion of the conjugate. where contact of the conjugate with the target cell results in delivery of the agent to the cell. followed by cell membrane healing as described above. Preferably. the conjugate includes a biological agent or a nucleic acid.

Thus. the second aspect involves a method of delivering a liposome-encapsulated biological agent or nucleic acid to a tissue containing both healthy cells and membrane-disrupted cells. the membrane disruption either existing naturally or having been induced specifically. The method includes contacting the tissue with an effective amount of a specific affinity reagent-liposome conjugate containing the biological agent or nucleic acid and targeted to membrane-disrupted cells of the tissue. Contact of the conjugate with the target cells results in delivery of the agent to the cells of the tissue and restoration of the integrity of the cell membrane.

In preferred embodiments of both aspects of the invention. the specific affinity reagent comprises an antibody. and the antibody may be specific for and bind to an intracellular antigen such as a cytoskeletal antigen. The tissue may comprise cardiac tissue. and the antibody may thus be specific for an intracellular antigen of cardiac tissue. The tissue may be cancerous or normal.

The invention also features methods of inhibiting cell death in cardiac tissue. comprising providing cardiac tissue containing cardiomyocytes having intracellular myosin exposed at the cell exterior; contacting the cardiomyocytes with a conjugate comprising an immunoliposome comprising an antibody specific for an intracellular antigen of cardiomyocytes for a time sufficient to allow the conjugate to adhere to the cardiomyocyte; and determining the viability of cells of the tissue. Preferably. the intracellular antigen comprises myosin.

The invention also features a method of delivering genetic material to a cell. comprising contacting a specific affinity reagent-liposome conjugate containing genetic material with a cell having a disrupted cell membrane for a time and in an amount sufficient to deliver genetic material to the cell and to prevent death of the cell; and determining the viability of the cell.

The invention also encompasses a method for enhancing in a mammal the effects of therapy that kills malignant cells in vivo. comprising the steps of: providing an immunoliposome specific for an internal cellular antigen present in degenerating neoplastic cells that is not present on the exterior of such cells. wherein the immunoliposome contains an antineoplastic agent; initiating therapy in a mammal to kill malignant cells in vivo. thereby causing some of the malignant cells to become necrotic; and administering the immunoliposomes to the mammal whereby the antibodies become bound to the internal cellular antigen present in necrotic malignant cells. thereby delivering the antineoplastic agent into the cells.

Preferably, the antineoplastic agent is selected from the group consisting of cytotoxic agents, toxins, radiosensitizing compounds, alpha-emitting radionuclides, beta-emitting radionuclides, antiproliferative agents and genes (for cytokines such as IL-2 and TNF). Preferably, the cells are killed with radiation, chemotherapy or immunotherapy.

As used herein, "specific affinity reagent" refers to any organic substance which has affinity for (i.e., binds to a substantially greater degree to) the target substance or molecule than for a non-target substance or molecule. As used herein, the term includes antibodies specific for certain organs, tissues, cells, or cellular antigens, particularly intracellular antigens, and also includes ligands for which an internal cellular receptor exists.

In preferred embodiments of both aspects of the invention, the specific affinity reagent may be an antibody, e.g., specific for an intracellular antigen, or a ligand that binds to an intracellular receptor. "Intracellular antigen" is meant to include antigens present in both neoplastic and normal cells, but not to antigens released into the general circulation upon cell death or to antigen on the exterior of living cells. "Tissue" refers to an agglomeration of cells that live in contact with each other in vivo, and may refer to both normal and abnormal tissues, including tumors, cancers, precancers, and neoplasms.

A "disrupted cell membrane" is defined herein as a cell membrane containing a physical disruption, e.g., a lesion, breach or hole through which the intracellular contents are accessible from the exterior of the cell. "Healthy" cells do not normally have disrupted cell membranes and their intracellular contents are not normally accessible from the external cellular environment; therefore, an "unhealthy" cell refers to a cell having a disrupted cell membrane, i.e., a cell that is in the early stages of the process of cell death.

As used herein, "necrosis" refers to the process that cells undergo as they die and involves a physical disruption of the cell membrane. Upon initiation of necrosis, the integrity of the cell membrane is lost as the membrane is dissolved to form a lesion. At the site of lesion, the cell membrane is gradually destroyed by biological, enzymatic and chemical degradation. At later stages of necrosis, more intracellular components, including nuclear antigens, structural elements in the cell, and organelles, eventually wash out, leaving a cell ghost, i.e., insoluble structural components of the cell and cell membrane. As used herein, cell death by necrosis is inhibited, prevented, or reversed upon sealing of a disrupted cell membrane by immunoliposomes.

A "salvageable" membrane-disrupted cell is a cell which can be contacted with immunoliposomes so as to reverse the dying process. Thus, "salvageable" cells refers to those cells which are in an early stage of necrosis, i.e., which possess some lesions or holes in the cell membrane, rendering one or more intracellular antigens accessible from the cell's exterior, but which still retain the cell nucleus and organelles. A salvageable cell will contain an intact nucleus and will not expose internal nuclear antigens to the cell exterior.

An "unsalvageable" cell is a membrane disrupted cell that has progressed beyond the stage of early necrosis, i.e., which has developed large membrane lesions and has lost structural elements in the cell such as cell organelles, and which may retain a cell ghost. An unsalvageable cell will expose internal nuclear antigens to the cell's exterior, and may have lost the nucleus altogether, and thus cannot be prevented from dying upon exposure to immunoliposomes.

A "healthy" cell includes cells that are not membrane-disrupted and thus their intracellular antigens are not exposed to the extracellular environment. Thus, contacting of a healthy cell with an immunoliposome specific for an intracellular antigen will not result in binding of the antibody portion of the liposome to that antigen.

An "unhealthy" cell refers to cells that contain a breach or disruption in the cell membrane severe enough to expose an intracellular antigen to the extracellular environment. Thus, contacting of an unhealthy cell with an immunoliposome specific for an intracellular antigen will result in binding of the antibody portion of the liposome to the intracellular antigen. "Delivery" of a biological agent or nucleic acid into a cell may be determined by direct or indirect methods, depending upon the substance delivered. For example, where a nucleic acid is delivered, viable cells may be assessed for the presence or absence of the nucleic acid (e.g., via hybridization) or for gene expression and detection of the expressed product.

A biological "drug" or "agent" includes both therapeutic agents and effector molecules. A "therapeutic agent" refers to, e.g., antineoplastic agents and other pharmaceuticals and genes (for tissue transformation or differentiation). An "effector molecule" refers to, e.g., cytotoxic molecules, radionuclides, imaging agents, reporter groups, etiologic agents of infectious diseases, metal chelating agents for radionuclide or paramagnetic metal ions, and genes (e.g., IL-2, TNF) which can mobilize the immune effector cells.

An "effective amount" refers to an amount sufficient to result in the desired effect at the target site; for example, where membrane sealing of a disrupted membrane is desired, an amount of immunoliposome sufficient to arrive at the target membrane lesion and inhibit cell death will be considered an effective amount. Alternatively, where a drug or gene is desired to be delivered to a target cell, tissue or organ, an amount of the drug or gene is loaded into an immunoliposome and an amount of loaded immunoliposome is delivered to the target site, the amounts in combination being sufficient to observe the desired effect upon delivery of the drug or gene to the site.

Therapeutic uses of the methods of the invention include therapeutic intervention after thrombolytic drug treatment, enhanced delivery of genetic material as for gene therapy, and improved delivery of drugs for cancer therapy.

Further features will become more fully apparent in the following description of the embodiments of this invention and from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings:

FIG. 1 is a schematic representation of the proposed mechanism of sarcolemmal repair;

FIG. 2 shows cardiac myocyte H9C2 viability as assessed by a trypan blue exclusion test according to a method of the invention;

FIG. 3 shows cardiac myocyte H9C2 viability as assessed by ^{3}H-thymidine uptake according to a method of the invention;

FIG. 4 shows liposome adherence to cardiomyocytes according to a method of the invention as assessed by fluorescence/confocal microscopy;

FIG. 5 shows liposome adherence to cardiomyocytes according to a method of the invention as assessed by fluorescence/confocal microscopy;

FIG. 6 shows liposome adherence to cardiomyocytes according to a method of the invention as assessed by fluorescence/confocal microscopy;

FIG. 7 shows liposome adherence to cardiomyocytes according to a method of the invention as assessed by fluorescence/confocal microscopy: and

FIG. 8 shows ultrastructure examination of the localization of silver grains containing immunoliposomes according to a method of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based on the discovery that immunoliposomes which are specific for a cellular antigen may be used for cell-targeting for the purpose of delivering a substance into the target cell or for inhibiting or reversing the process by which cells undergo death.

Cell Death

Necrosis and apoptosis are processes by which cells degenerate and eventually die. Cell death in an organ of the body may be the result of ischemic, inflammatory, immunologic and/or toxic insult to the tissue. Depending upon the location and severity of cell death, the consequences may be benign or significant enough to result in severe illness or death. Necrosis involves a physical disruption of the cell membrane due to oxygen deprivation or other chemical means. Upon initiation of necrosis, the integrity of the cell membrane is lost as the membrane is dissolved to form a lesion. At the site of lesion, the cell membrane may be gradually destroyed by phagocytosis and enzymatic degradation, and intracellular components, including nuclear antigens, structural elements in the cell, and organelles, eventually wash out.

Reperfusion injury is also believed to result in significant cell death. When blood is restored to a vascular area during reperfusion, oxygen is returned to an oxygen-deprived area and superoxide radicals may form, causing damage to cell membranes.

Cell death or viability may be determined according to the invention by one of several different methods. Dead cells can be distinguished from viable, i.e., live cells via conventional trypan blue exclusion, with dead cells appearing blue (e.g., using reagents as described in the catalogue of Sigma Chemical Co., 1995, p. 1681). In addition, cell death is determined by culturing cells in a culture dish or flask, wherein dead cells will float freely in the medium and live cells will adhere to the walls of the flask, as taught herein. Alternatively, cell viability may be determined via ^{3}H-thymidine uptake by standard methods well known to those of ordinary skill in the art.

Liposomes

Liposomes are composed of a bilayer lipid matrix that wraps around an aqueous volume, thus isolating it from the external medium. The central aqueous core may vary in diameter from 20 nm to as much as 2–3 micrometers. The term "liposome", as used herein, is also intended to encompass liposomes which are composed of several (e.g., 2–3) concentric bilayers which define several individual aqueous compartments. Thermodynamically, liposomes have minimum free energy as long as the density of the phospholipids in each monolayer of the bilayer structure is the same.

Liposomes useful in the invention are composed of phospholipid molecules. A phospholipid molecule has a polar head group and two nonpolar, hydrophobic fatty acyl chains. In an aqueous environment, the most energetically stable form for phospholipids is within structures that allow the fatty acyl chains to avoid contact with water. A lipid bilayer is one such structure. Many phospholipids, when dispersed in water, spontaneously form lipid bilayer structures. Lipid bilayer structures useful in the invention are preferably circular structures.

The polar head group of the phospholipid molecule may include choline, e.g., lecithins (phosphatidylcholines) and sphingomyelins. Such molecules may also include amino groups, e.g., phosphatidylserine and phosphatidyl ethanolamine. Other polar head groups may include phosphatidylglycerol, phosphatidylinositol and cardiolipin.

The invention contemplates the use of immunoliposomes to inhibit or reverse cell death, or for delivering an agent to a target cell. An immunoliposome will include a liposome component, as described above, conjugated via its polar head group to the carboxy terminus of an immunoglobulin molecule. Fusion of an immunoliposome with the cell membrane will occur because most cell membranes are composed of protein and phospholipid bilayers. Immunoglobulins will be used for targeting liposomes to dying cells.

Mechanism of Action

Without being bound to any one theory, it is hypothesized that cell death in membrane-disrupted cells is inhibited or reversed as a result of immunoliposome-induced sealing of the cell membrane at the site of disruption. FIG. 1 is a schematic representation of the proposed mechanism of sarcolemmal repair. In FIG. 1, a portion **10** of a normal cardiac cell is shown, in which the cell membrane lipid bilayer **12** is intact and overlies myosin **14**. FIG. 1 also depicts a disrupted cell membrane **16** in which the lipid bilayer **18** is breached and myosin strands **20** are exposed. The hypothesis contemplates specific targeting of an immunoliposome to the site of breach **18**, the specificity being conferred via antibody/antigen interaction. That is, immunoliposomes **22** containing antibodies specific for myosin (anti-myosin antibodies **24**) specifically target and bind to the exposed myosin in the region of breach, thus anchoring the immunoliposome to the disrupted cell at the site of breach **26**. Such anchoring is believed to inhibit cell death by effectively plugging the membrane lesion, thus preventing the continued process leading to cell death, e.g., the intracellular contents from washing out. Fusion **28** of the immunoliposome to the disrupted cell membrane may follow anchoring, thus allowing interaction between anti-myosin antibodies and myosin over a greater area of the cell, and initiate repair of the membrane breach. Irrespective of the exact mechanism involved, the intraliposomal contents can also be delivered ultimately into the intracellular space. Therefore, the method of the invention is amenable as a general delivery technique, e.g., for a biological agent or nucleic acid.

Preparation of Liposomes and Immunoliposomes

Liposomes and immunoliposomes may be prepared according to a variety of techniques, e.g., detergent dialysis or the formation of a water-in-oil emulsion, slow swelling in nonelectrolytes, dehydration followed by rehydration, dilution or dialysis of lipids in the presence of chaotropic ions, and mechanical preparation techniques such as freeze-thaw cycling.

Removal of detergent molecules from aqueous dispersions of phospholipid/detergent mixed micelles represents one way of producing liposomes (see J. Biol Chem. 246:5477 (1971) herein incorporated by reference). As the detergent is removed, the micelles become progressively richer in phospholipid and finally coalesce to form closed, single bilayer vesicles. Detergents commonly used for this purpose include bile salts and octylglycoside. Because this method does not involve the use of organic solvents and

sonication, it is particularly useful for entrapping macromolecules, such as proteins and nucleic acids, which are sensitive to the presence of organic solvents or are structurally altered by sonication.

Another method of preparing liposomes is the reverse phase evaporation method detailed in U.S. Pat. No. 4,235,871, which is incorporated herein by reference. Liposomes prepared by this method have a typical average size of about 2–4 microns and are predominantly oligolamellar, that is, contain one or a few lipid bilayer shells.

Liposomes may also be prepared via hydration in the presence of a solvent. Multi-lamellar vesicles (MLVs) with high encapsulation efficiency can be prepared by hydrating the lipids in the presence of an organic solvent. The two phases are emulsified by vigorous mixing (vortexing) and then the organic phase removed by passing a stream of nitrogen gas over the emulsion. As the solvent evaporates, liposomes form in the aqueous phase.

Mechanical preparation methods, e.g., shaking by hand, sonication, French pressure freeze-drying, membrane extrusion, freeze-thawing, changing pH, calcium inducing, and micro emulsion techniques, have been used for the preparation of liposomes. In essence, a mixture of vesicle-forming lipids in a volatile organic solvent is deposited on the surface of a round bottomed flask and the solvent is removed by rotary evaporation under reduced pressure. Vesicles ranging in size from one-tenth to tens of microns form spontaneously when an excess volume of aqueous buffer is added with agitation to the dry lipid. The drug to be entrapped may be included either in the lipid film (for a lipophilic drug) or in the aqueous hydration medium (for a hydrophilic drug).

Methods for controlling the size of liposomes are various and include extrusion and homogenization. One effective sizing method involves extruding an aqueous suspension of the liposomes through a series of polycarbonate membranes having a selected uniform pore size in the range of 0.2–0.6 micron, typically 0.1–0.2 micron. The pore size of the membrane corresponds roughly to the largest sizes of liposomes produced by extrusion through that membrane, particularly where the preparation is extruded two or more times through the same membrane. Extrusion of liposomes can also be performed through an asymmetric ceramic filter, as taught in U.S. Pat. No. 4,737,323, herein incorporated by reference. Other methods of reducing particle size include application of high pressures to the liposomes, as in a French Press, and homogenization of the liposomes.

Immunoliposomes

Antibodies, and in particular monoclonal antibodies, are the focus of intense interest in the field of cancer research. Antibodies have been developed to cell-surface antigens for a number of malignancies, but are useful only in restricted categories of tumors. Techniques are known for conjugating such antibodies to pharmacologically active agents or to labels to permit diagnosis, localization, and therapy directed toward such tumors.

Recent research has led to the identification of unique nuclear antigens and development of monoclonal antibodies thereto. See, e.g., Epstein and Clevenger, Exp. Cell. Res. 151:194 (1984) and J. Histochem. and Cytochem. 32:757 (1984). Such antibodies have been labeled and used to identify structures in the nucleus.

Liposome targeting based on antibody/antigen recognition has been utilized in the prior art in the development of targeted delivery systems for delivery of various bioactive agents to a target site. Antibody-directed liposomes, or immunoliposomes, are used for this purpose. Antibody molecules are predominantly hydrophilic compounds with no affinity for the hydrophobic liposome membrane.

As used herein, an immunoliposome comprises a liposome conjugated to an immunoglobulin molecule. Generally and as used herein, a liposome/immunoglobulin conjugate comprises an immunoglobulin molecule linked via direct or indirect means and either covalently or noncovalently to the phospholipid molecule. Generally, about 1 in every 200 phospholipid molecules of the liposome will be linked to an antibody molecule, with an acceptable range being 1 in every 20–20,000 phospholipid molecules of the liposome.

Where enhancement of specificity of the immunoliposome for the target site is desired, the immunoliposome may include antibodies of several different specificities, each cognate antigen being found at the target site. Such multiple specificity may also be conferred using bifunctional or trifunctional antibodies (see, e.g., U.S. Pat. No. 5,237,743, hereby incorporated by reference).

Methods are known in the prior art for preparing immunoliposomes. Immunoliposomes are prepared, for example, by adsorption of proteins (e.g., immunoglobulin) on the liposomal surface; incorporation of native protein into the liposome membrane during its formation (e.g., by ultrasonication, detergent dialysis or reverse phase evaporation); covalent binding (direct or via a spacer group) of a protein to reactive compounds incorporated into the liposomes membrane; noncovalent hydrophobic binding of modified proteins during liposome formation or by the incubation with preformed liposomes); and indirect binding, including covalent binding of immunoglobulin protein via a polymer to the liposome (see Torchilin, V. P. CRC Critical reviews in Therapeutic Drug Carrier Systems, vol. 2(1), hereby incorporated by reference).

Immunoliposomes may be prepared according to the following procedure.

1. Covalent Coupling of Antibody with NGPE.

0.6 mg N-Glutaryl phosphatidylethanolamine (NGPE) was dissolved in 0.5 cc 2-[N-morpholino]ethanesulfonic acid hemisodium salt (MES) buffer (in 0.016M octylglucoside in 50 mM MES). After the addition of 4.8 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 6 mg N-hydroxysulfosuccinimide (HSSI), the resulting mixture was incubated at room temperature for 5 min. The antibody solution (containing antimyosin antibody 2G42D7, described below, or other antibody) was then added (0.36 mg/ml). The pH of the mixture was then adjusted to 8.0 with 1M NaOH. The reaction mixture was incubated at 4° C. for 8–12 hour with mixing. The resulting NGPE-antibody conjugate was then dialyzed overnight against PBS, pH 7.4 to remove octylglucoside and other excess reagents.

2. Preparation of Immunoliposomes by detergent dialysis.

Liposomes were prepared from a mixture of egg phosphatidylcholine (PC) and cholesterol (Ch) in chloroform in the molar ratio 1:1. The lipid mixture (30 mg PC/17.96 mg Ch) was dried with argon, then vacuum dried for 2 hour and resuspended in 4 cc phosphate buffered saline (PBS) containing 0.016M octylglucoside with brief ultrasonication. The solution of NGPE-modified antibody (0.7 mg/ml) was added to solubilized lipids. The mixture was dialyzed overnight against PBS (pH 7.4) to remove detergent. The resulting liposomes were extruded through a Nucleopore filter (0.6, 0.4, and 0.2 μm). The same method was used for preparation of liposomes without NGPE-antibody solution.

3. Preparation of Fluorescent Liposomes.

Liposomes were prepared from a mixture of egg phosphatidylcholine (PC), cholesterol (Ch) and L-α

phosphatidylethanolamine-N-fluorescein (FL) in chloroform. The lipid mixture (30 mg PC/17.96 mg CH/0.1 mg FL) were dried with argon, then vacuum dried for 2 hour and resuspended in 4 cc PBS containing 0.016M octylglucoside with brief ultrasonication. The solution of NGPE-modified antibody (0.7 mg/ml) was added to the solubilized lipid. The mixture was dialyzed overnight against PBS (pH 7.4) to remove detergent. The resulting liposomes were extruded through a Nucleopore filter (0.6, 0.4, and 0.2 μm). The same method was used for liposomes without NGPE-antibody solution.

4. Preparation of electron-dense Antimyosin Immunoliposomes.

For ultrastructural characterization of antimyosin liposome sealing, electron-dense antimyosin immunoliposomes were prepared. Lipid mixture (30 mg PC/17.96 mg Ch) was dried with argon then vacuum dried for 2 hour and resuspended in NGPE-antibody solution (0.7 mg/ml). Two mg $AgNO_3$ was added to the solution, which was vortexed and sonicated slightly. The resulting liposomes were extruded through filter (0. 6, 0.4, 0.2 μm). The mixture was dialyzed overnight against water. 200 μl 0.145M NaCl was added and the mixture was exposed to light for 1 hour. For processing of electron microscopic characterization, liposome containing medium was removed, cells were washed with PBS (pH 7.4). The cells were then fixed with 2.5% glutaraldehyde plus 2% paraformaldehyde in 0.1M Na cacodylate buffer pH 7.2. Fixed and pelleted cells were dehydrated in an alcohol series. Ultrathin sections contrasted with uranyl acetate and lead citrate was studied in transmission electron microscopy (TEM).

Loading of Immunoliposomes

Loading of compounds into liposomes may be achieved by one or more of a variety of active and passive methods. Passive loading by entrapment is employed for those compounds which are therapeutically active at relatively low drug doses, for drugs which are highly soluble in aqueous solutions, or for DNA which is expressed at a high efficiency in a target cell. It has been found for certain hydrophobic drugs, that the highest concentration of encapsulated material which can be achieved by passive loading is limited by their low intrinsic water solubility. The concentration of hydrophobic drug that can be accommodated in the liposome will depend on drug/lipid interactions in the membrane, but is generally limited to a drug concentration of less than about 20 mcg drug/mg lipid. In the case of amphipathic drugs having a positive charge, it has been found that inclusion of 20–30 mole percent of an anionic phospholipid in the liposomal membrane results in increasing the loading factor significantly through formation of an "ion pair" complex with the negatively charged phospholipid at the membrane interface.

Loading of high concentrations of drugs in liposomes may require active loading methods, e.g., as described in U.S. Pat. No. 5,129,549, herein incorporated by reference, in which a chemical gradient is created across the liposome membrane that results in trapping of the drug in the internal aqueous phase of the liposome.

Liposome/drug formulations are characterized by measurements of particle size, lipid concentration, and pH by standard methods as described above. Drug incorporation into the composition may be determined by inclusion of radiolabeled tracer in the composition. The amount of liposome-entrapped drug is then determined by gel permeation chromatography using BioRad A-15M resin. The liposomal drug fraction is calculated from the amount of radiolabel present in the void volume of the column, and the percentage of liposomal drug from the ratio of label eluting in the void volume to the remaining label eluting from the column.

Preparation of Antibodies

Immunoglobulin molecules useful in the invention include whole antibody, or any antibody fragment, for example, a F(ab')2, Fab, and/or an Fv fragment of an antibody molecule. In addition, any variable region specificity of an antibody molecule is useful according to the invention.

A F(ab')2 fragment is that portion of an antibody molecule which contains the complete antigen-combining site, consisting of two light chains and part of each heavy chain, and is produced by enzymatic digestion, e.g., using pepsin, such that the heavy chain disulfide bonds remain intact in the F(ab')2 fragment. A Fab fragment consists of a single light chain and a part of a heavy chain disulfide bonded together. Fab is produced by enzymatic digestion, e.g., using papain, such that about one-half the F(ab')2 antigen binding fragment is generated. An Fc fragment is that portion of an antibody that is responsible for binding to antibody receptors on cells and the Clq component of complement. The Fc fragment is the portion of the antibody molecule that remains after papain digestion. An Fv fragment is that portion of an antibody consisting of the variable region of a Fab fragment.

Antibodies useful in the invention may be obtained through conventional polyclonal or monoclonal antibody preparation techniques. Antigen may be obtained from cells of the species toward which the antibodies are to be directed. Such species are preferably vertebrate, more preferably mammalian, and most preferably human. For antibodies directed toward human intracellular antigens, immortal cell lines represent a convenient source of such antigen.

To generate monoclonal antibodies, murine spleen cells from immunized animals are fused with an appropriate myeloma cell line. Fused cells are cultured in selective growth medium to establish hybridoma colonies, each colony secreting an antibody of interest. Culture supernatants from each colony are then tested for antibody specificity. Positive cultures are identified and expanded. See Kohler et al., Nature 256:495 (1975), hereby incorporated by reference.

Target Antigens

Antigens useful in the invention include intracellular antigens, preferably cytoskeletal antigens, for specific cell types that are to undergo treatment. These cell types include any type of cell that is useful as a target for cell salvage or for gene therapy, including but not limited to cardiac muscle cells, skeletal muscle cells, liver cells, and spleen cells. The antigens include intracellular antigens, and antigens which are more available for binding when a cell, tissue, or organ is in a diseased or unhealthy state than in a healthy state. Intracellular cytoskeletal antigens will be particularly useful for anchoring of the immunoliposomes used to plug a membrane lesion.

Antibodies that are useful for targeting such antigens include but are not limited to: anti-myosin antibody (for example, R11D10, Centocor, Malvern, Pa. or 2G42D7 (described below)), anti-actin antibody, anti-cytoskeletal antibodies, anti-histone antibody, anti-nuclear antibodies, anti-endoplasmic reticulum antibody, and anti-protein kinase C antibody. All such antibodies are publicly available through the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A.

In order to screen for monoclonal antibodies that bind specifically to cells undergoing the process of cell death, i.e.,

that bind to intracellular antigens, equal aliquots are prepared from cell cultures, one aliquot containing healthy cells and another aliquot containing cells that have been subject to several cycles of vigorous pipetting to disrupt cell membranes. Cells having undergone membrane disruption will uncover intracellular components for antibody recognition that would not normally be available for such recognition in the absence of membrane lesions. The ability of a monoclonal antibody from each test aliquot to bind the healthy or membrane disrupted cells is then quantitatively measured, e.g., by radioimmunoassay or direct or indirect immunofluorescence screening techniques. Specificity for intracellular antigens is determined by comparing binding to the test aliquots. Those antibodies which bind only to the membrane disrupted cells, and do not bind to healthy cells, can be considered good candidates for possessing specificity to intracellular antigens.

Because even a healthy cell culture may contain a relatively large proportion of necrotic cells, an antibody that is specific for an intracellular antigen may exhibit some binding to the healthy cell culture. It should also be recognized that even though the antibody may not be specific to any surface protein or antigen of the cell line employed in the screening process, certain tumor cell lines (such as histiocytic cell lines) have surface components that exhibit generalized binding of immunoglobulins.

Therefore, it is preferable to perform a second screening step for antibodies to intracellular antigens, as identified above, the second step being to screen the candidate antibodies for comparative binding to similar in vivo cells that are healthy versus the same in vivo cells that have been cell membrane disrupted.

Labeling and Labels Useful in the Invention

The various labels listed below need not be limited to labeling of antibodies. Instead of antibodies, other carriers (synthetic or natural) may be used as recipients of the labels.

(1) Radiolabels

For imaging purposes, any of the well-known medical radionuclides can be used. Suitable radionuclides include Tc-99 m, I-123, I-125, In-111, In-113 m, Ga-67, or other suitable gamma-emitters.

For example, iodination, may be accomplished using the chlormine-T method described in Mills et al., Hybridoma 5: 265–275 (1986). This technique may be used to effect iodination to render the antibody radiopaque or to attach a radionuclide, such as I-125 or I-131.

Other radionuclides may be attached to the antibodies in question through chelation with benzyl EDTA or DPTA conjugation procedures. Still other suitable techniques include the iodogen method disclosed in Pimm et al., Int. J. Cancer 30:75 (1982), and direct iodination with radioactive sodium iodide.

(2) Radiopaque Materials

Radiopaque materials also may be used to label the antibodies. Suitable radiopaque materials are well known and include iodine compounds, barium compounds, gallium compounds, thallium compounds, and the like. Specific examples of radiopaque materials include barium, diatrizoate, ethiodized oil, gallium citrate, iocarmic acid, iocetamic acid, iodamide, iodipamide, iodoxamic acid, iogulamide, iohexol, iopamidol, iopanoic acid, iotasul, iotetric acid, iothalamic acid, iotroxic acid, ioxaglic acid, ioxotrizoic acid, ipodate, meglumine, metrizamide, metrizoate, propyliodone, and thallous chloride.

(3) Magnetic Resonance-Enhancing Materials

Materials that can be detected by or that enhance the effects of magnetic resonance imaging equipment also may be conjugated to the antibodies. Suitable conventional magnetic resonance-enhancing compounds include gadolinium, copper, iron, and chromium. It is preferred that these metal atoms be prepared in the form of a conventional organometallic chelates, which are then bound to the antibody.

Conjugation of Labels and Therapeutic Compounds to Antibodies or Other Carriers

Numerous techniques suitable for binding various molecules to antibodies or other carriers have been established. Numerous techniques are available for attaching various molecules, enzymes and proteins to antibodies. For example, many carboxylic acid-containing compounds (such as methotrexate) can be conjugated to immunoglobins through an active ester intermediate, formed, e.g., by reacting the compound with N-hydroxysuccinimide and dicyclohexylcarbodiimide. See, T. Deguchi, et al., Effect of Methotrexate-Monoclonal Anti-Prostatic Acid Phosphatase Antibody Conjugate on Human Prostate Tumor, Cancer Res. 46:3751–3755 (1986). Others, such as chlorambucil, will bind directly to the antibodies at low pH. See, e.g. T. Ghose, et al., Immunochemotherapy of Human Malignant Melanoma with Chloroambucil-Carryine Antibody, Europ. J. Cancer 11:321–326 (1975).

Amino sugar-containing drugs such as adriamycin and daunomycin may be covalently bound to antibodies by periodate oxidation of the drug, followed by linking of the oxidized drug to the immunoglobulin and subsequent reduction of the product with sodium borohydride. E. Hurwitz, et al., The Covalent Binding of Daunomycin and Adriamycin to Antibodies, Cancer Res. 35:1175–1181 (1975).

Conventional techniques also exist for binding biologically active molecules or other proteins to antibodies. Free thiol groups may be introduced into the antibody by reacting antibody with N-succinimidyl-3-(2-pyridyldithio) propionate (SPOP) to introduce 2-pyridyl disulphide groups. The protein to be bound to the antibody is incubated with SPDP. Upon mixing the SPDP-modified protein with the antibody containing free thiol groups, the two materials become bound.

Other known techniques, such as the use of dextran T-10 spacers to increase the number of drug moieties linked to antibody molecules can be employed, as can mixed anhydride methods of drug conjugation. The compound 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (ECDI) may be used to bind amino-containing drugs to the carboxyl groups of antibodies. Alternatively, glutaraldehyde may be used for crosslinking between free amino groups of the antibody and amino groups in the compound to be conjugated thereto.

Inhibition or Reversal of Cell Death in Cardiac Cells

Cell death in an organ such as the heart may be highly detrimental due to the inability of certain cells of the heart, e.g., cardiomyocytes, to proliferate and thus replace themselves. Cardiomyocytes are highly differentiated cells which are not capable of division.

The cardiac protein myosin is well known. This protein is an intracellular muscle protein found inside cardiac cells, but not on the cell wall. Myosin-specific antibodies have been developed and have been labeled for in vivo imaging of heart tissue damaged by myocardial infarction. (See, Khaw et al., J. Clin. Invest. 58:439, 1976).

Cardiac necrosis may be inhibited or reversed by administering immunoliposomes specific for, e.g., cardiac myosin, to a patient in an amount and for a time sufficient to inhibit or reverse cell death. Immunoliposomes may be administered intravenously as a single dosage, intermittently for, e.g., 1–10 min., or continuously over a period of hours or

days, as required. Administration of myosin-specific immunoliposomes may be especially useful, e.g. during reperfusion therapy to off-set the injury to tissue that occurs during such therapy. Therapeutic immunoliposomes may also be delivered by intracoronary infusion directly into the region at risk as a co-therapeutic regimen to angioplasty or thrombolysis.

Therapeutic Agents

Therapeutic agents which may be encapsulated and thus used according to the invention for delivery to a target cell are presented in Table I. Table I is a list of representative useful agents and is not meant to be exhaustive. Generally, any therapeutic agent that is encapsulatable in a liposome and is therapeutically effective when used for targeted delivery is encompassed by the inventive methods. Also, as presented in Table I, antimyosin immunoliposomes can particularly be used for targeting of drugs likely to be of benefit for ischemically compromised myocardium.

TABLE I

Drug	Disease treated or effect desired	Probable benefit rendered by liposomes
Amphotericin B	Systemic fungal infections	Significantly greater efficiency
Doxorubicin	Cancer	Reduced cardiotoxicity, immunosuppression emesis, alopecia; enhanced efficacy
Cisplatin	Cancer	Reduced nephrotoxicity and emesis. New indications in leukemias, Not cross-resistant with cisplatin lymphikomas and liver metastases.
Vincristine	Cancer	Reduced neurotoxicity; enhanced efficacy
Muramyltripeptide	Cancer-immunomodulator	Only acceptable carrier; targets to macrophages
Gentamicin	Gram-negative pneumonia; other gram negative infections	Reduced nephrotoxicity; significantly improved efficacy
Streptomycin	Bovine brucellosis	Only cure
Indomethacin	Arthritis	Reduced gastric toxicity
Bovine somatotropin	Increased milk production	Infrequent dosing, e.g., once every two weeks
Epithelial growth factor	Aid in wound healing	Infrequent dosing
Pilocarpine	Glaucoma	Reduced dosing, e.g., one drop per day
Tear components	Dry eye	Increased comfort; longer lasting
Indium-111	Tumor-imaging	Preferential accumulation in tumor
Fibroblastic growth factor-β	Neovascularization in the infarcted myocardium	Likely to be of benefit in post infarction period
Angiogenic factors	Neovascularization in the infarcted myocardium	Likely to be of benefit in post infarction period
High energy substrates for the myocardium	Supplemental for depleted source of energy	Likely to be of benefit in post infarction period
Antioxidants, e.g., superoxide dismutase β-hydroxy anisole	For prevention of free radical injury	Likely to be of benefit in reperfusion of ischemic myocardium
Cytokines, e.g., EGF, IL-1, IL-6	Stimulation of myocardial cell	Likely to be of benefit in ischemic myocardium
Contrast agents e.g., urografin	For production of specific contrast agents	Likely to be of radiodiagnostic use for myocyte necrosis

[The first part of Table I is drawn from: Ostro, M. J., Introduction. In Liposomes: From Biophysics to Therapeutics. Ed.: Ostro, M. J. Marcel Decker, Inc., New York, 1987.]
[The second part of Table I is drawn from research leading to the invention, as described herein.]

A large number of antineoplastic agents are known which can be loaded into immunoliposomes and used as contemplated in the present invention. In addition to the above-listed agents, antineoplastic agents may include folate inhibitors, pyrimidine analogs, purine analogs, alkylating agents, antibiotics, and radiosensitizing compounds. Specific examples of such antineoplastic agents include acivicin, aclarubicin, acodazole, adriamycin, ametantrone, aminoglutethimide, anthramycin, asparaginase, azacitidine, azetepa, bisantrene, bleomycin, busulfan, cactinomycin, calusterone, caracemide, carboplatin, carmustine, carubicin, chlorambucil, cisplatin, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin, dezaguanine, diaziquone, doxorubicin, epipropidine, etoposide, etoprine, floxuridine, fludarabine, fluorouracil, fluorocitabine, hydroxyurea, iproplatin, leuprolide acetate, lomustine, mechlorethamine, megestrol acetate, melengestrol acetate, mercaptopurine, methotrexate, metoprine, mitocromin, mitogillin, mitomycin, mitosper, mitoxantrone, mycophenolic acid, nocodazole, nogalamycin, oxisuran, peliomycin, pentamustine, porfiromycin, prednimustine, procarbazine hydrochloride, puromycin, pyrazofurin, riboprine, semustine, sparsomycin, spirogermanium, spiromustine, spiroplatin, streptozocin, talisomycin, tegafur, tenoposide, teroxirone, thiamiprine, thioguanine, tiazofurin, triciribine phosphate, triethylenemelamine, trimetrexate, uracil mustard, uredepa, vinblastine, vincristine, vindesine, vinepidine, vinrosidine, vinzolidine, zinostatin and zorubicin.

In addition, alpha-emitting and beta-emitting radionuclides may be used. Such compounds include I-131, Yt-99, Cu-67, Au-198, Re-186, Re-188, P-32, and other cytotoxic radionuclides.

According to the invention, immunoliposomes also may be the carriers of biological response modifiers, including cytokines such as interleukin-2, vasodilators, any of the interferons, tumor necrosis factor, and the like.

Yet another category of compounds that may be transported by immunoliposomes according to the present invention are toxins such as ricin, tetanus, diphtheria, abrin, gelonin, mistletoe, and other materials capable of causing localized necrosis.

Immunoliposomes for Delivery of DNA to Membrane-damaged Cells

Immunoliposomes prepared according to the invention can be used for delivery of DNA to a target cell in two unique ways.

1. Shove and Seal delivery: Intracellular DNA delivery using immunoliposomes seeking intracellular targets followed by effective salvage of the cells.

Immunoliposomes containing DNA may be prepared so as to specifically target intracellular antigens in cells having damaged cell membranes. One immunoliposome/antibody conjugate exemplified herein is specific for the intracellular antigen myosin; however, the invention encompasses the use of immunoliposomes/antibody conjugates in which the antibody is specific for and thus targets any intracellular antigen, e.g., cytoskeletal or contractile proteins.

The net delivery of gene copies from the immunoliposome to the intracellular milieu will be significantly higher due to the abundance of the intracellular antigen concentration. Concomitant with intracellular gene delivery, liposomes effectively seal the breaches in the cell membrane, and thus maintain cell viability.

2. Local depot delivery: Immunoliposomes using intracellular targets of irreversibly injured cells as anchors for gene delivery to surrounding tissues.

DNA delivery also can be targeted to irreversibly damaged cells, i.e., cells that are beyond the stage where they can be salvaged via liposomes sealing. Immunoliposomes target an intracellular antigen of the irreversibly damaged cell and become anchored to the intracellular antigen. The DNA-loaded liposomes can then release the gene fragments around the anchor and free interstitial DNA fragments will be avidly adsorbed by various interstitial cells.

For example, DNA may be delivered to irreversibly damaged myocardium. If cells are targeted in acute myocardial infarction, surrounding cells which pick up DNA such as myoD and myogenin, as reported in Taylor, J. of NIH Research 6:57–61 (1994), may upon integration and expression of the foreign DNA develop as skeletal mycocytes, thus restoring contractile elements to the otherwise fibrous scar. Thus, the mode of DNA delivery according to the invention provides noninvasive and effective delivery to a partially damaged target tissue.

Both the "shove and cork delivery" and "local depot delivery" methods can also be applied to treatment of diseases such as cancer or cystic fibrosis by selecting appropriate DNA coding sequences for targeted delivery. For example, cytokines and/or growth factors may be delivered to cancer cells. Tumors contain many micronecrotic centers, i.e., areas of membrane-damaged cells. Immunoliposomes containing DNA encoding TNF or IL-2 may be targeted to cytoskeletal structures in tumor cells via micronecrotic centers in the tumor. Delivery of the genetic construct to the micronecrotic center would also initiate repair and sealing of the damaged cell membrane. The revived cell can then begin to produce the necessary cytokines in situ to initiate gene therapy. The local depot delivery approach may also be used to target DNA-containing immunoliposomes to tumor associated antigen for anchoring the immunotherapeutic liposomes. Thus, the above-described two modes of gene therapy, i.e., using immunoliposomes for delivery of DNA to damaged but salvageable cells or to damaged and unsalvageable cells, may initiate gene therapy for a variety of therapeutic applications, including cardiovascular applications, and to tumor gene therapy.

Chemotherapy using Loaded Immunoliposomes

Modern techniques for nonsurgical treatment of cancer include both clinical and experimental techniques involving chemotherapy, radiation therapy, a combination of chemotherapy and radiation therapy, and immunotherapy. In each instance, the object of the therapy is to kill the malignant cells. Antineoplastic agents presently or potentially useful in such therapy include cytotoxic drugs, biological response modifiers, radiosensitizing compounds, toxins, and radionuclides.

Loaded immunoliposomes also may be used in cancer chemotherapy by delivering a radioactive agent to micronecrotic areas of a target site, e.g., a tumor, using immunoliposomes containing a labeled agent prepared as disclosed herein, or by delivering an immunoliposome containing an antineoplastic agent to a target site.

Antineoplastic agents useful according to the invention include but are not limited to cytotoxic agent, toxin, biological response modifier, radiosensitizing compound, alpha-emitting radionuclide, beta-emitting radionuclide, antiproliferative agent.

Diagnostic Procedures Using Immunoliposomes

One diagnostic procedure of the present invention involves diagnosing sites of necrosis in an organ or tissue. This procedure utilizes immunoliposomes specific for intracellular antigens and containing a diagnostic agent, e.g., a detectable molecule such as an imaging agent. One example of such an agent is a gamma-emitting radionuclide of the type previously discussed. The radionuclide may be attached to a convenient carrier molecule, such as a chelating polymer. The radionuclide-containing immunoliposome is injected (preferably intravenously) into a patient suspected of containing an organ or tissue that is undergoing cell death; for example, a patient who has received chemotherapy, radiation therapy, or both. This procedure is preferably carried out at least one or two days after the initiation of the therapy, in order to permit resultant necrosis of the neoplastic tissue to advance to a sufficient point that reasonable numbers of necrotic cells are present. Between 30 minutes and 3 days following administration of the labeled antibody, an appropriate scintigraphic imaging technique is employed to image the label that is localized in necrotic tissue. Suitable imaging techniques include gamma cameras and SPECT (single photon emission computed tomography) techniques.

One alternative imaging technique is radiographic imaging. In this technique, immunoliposomes specific for intracellular antigen that has been labeled with a radiopaque material is injected a suitable time after initiation of chemotherapy or radiation therapy. After the antibody has localized at the areas of necrotic tissue, radiographic imaging is performed. Other suitable techniques include CAT (computed axial tomography) scans, fluoroscopy and conventional X-ray imaging.

Therapeutic Procedures Using Immunoliposomes

By loading immunoliposomes of the present invention with therapeutic, e.g., antineoplastic compounds, those therapeutic agents may be delivered directly to the neoplasm, with greatly reduced systemic effect. Two approaches may be employed; first, as augmentation therapy following use of an existing therapeutic modality that may

have killed all or part of the tumor; and second, as a primary de novo mode of therapy focusing on the neoplastic cells already showing some degree of abnormal permeability.

In the augmentation approach, tumor necrosis is initiated by any conventional technique, such as chemotherapy, immunotherapy, radiation therapy, or the like.

After initiation of such therapy, necrosis begins in the tumor mass. At this point (usually at least two days after initiation of the primary therapy), immunoliposomes containing the therapeutic compound, preferably an antineoplastic agent, are administered to the patient. Intravenous administration is preferred, although direct injection in the vicinity of the tumor is also contemplated.

Following administration of the loaded immunoliposomes, the immunoliposomes become bound to necrotic areas in the tumor mass, and deliver the antineoplastic agent to disrupted cells.

If a lethal amount of antineoplastic agent is delivered to a necrotic area, healthy, viable tumor cells surrounding this area may be rendered necrotic, enabling additional amounts of antineoplastic agent to penetrate the newly necrotic area. In this way, a diffusion effect may be possible with destruction of the tumor cells proceeding radially from necrotic to healthy tumor tissue. To achieve this diffusion effect, a cytotoxic agent such as a beta-emitting or an alpha-emitting radionuclide may be used.

In the second approach (primary therapy), the method is exactly as described above, except that the need for prior treatment with some other modality (to create an initial population of necrotic cells) is obviated.

The amount of antineoplastic agent-loaded immunoliposome administered to the patient will vary depending on the antineoplastic agent used and the size of the tumor. However, in general, the dosage is selected to administer a total dose of antineoplastic agent that is equal to or less than the conventional therapeutic dosage of the particular agent selected. It is preferred that the total dosage be between 1% and 50% of the conventional therapeutic dosage, and it is most preferred that the dosage be between 2% and 25% of the conventional therapeutic dosage of the compound. However, as with all cancer therapy, the optimum dosage will be determined by the treating physician based on the individual patient's response to the therapy and the side effects resulting therefrom.

Immunoliposomes are Efficient Sealers of Cell Membrane Lesions

Immunoliposomes are efficient sealers of cell membrane lesions, and thus are useful to protect membrane-damaged cells from further injury and death. Experimental evidence of efficient sealing by immunoliposomes of membrane-damaged cells is as follows. The examples provided herein utilize cardiac cells in a model system which mimics damage to cardiac cells as may occur in heart diseases.

H9C2 cardiomyocytes from the American Type Culture Collection (ATCC) in culture were subjected to hypoxic injury. Hypoxic myocytes were then incubated with one of antimyosin immunoliposomes, nonspecific antibody immunoliposomes, and liposomes containing no antibody. Control cardiomyocytes were incubated in regular medium without any liposomes or antibodies, and another set of controls was kept under normoxic culture conditions. After 24 hours of incubation at 37° C., myocyte survival/viability was assessed as a percentage of the total number of cells in each culture flask by trypan blue exclusion (FIG. 2) or ^{3}H-thymidine uptake (FIG. 3). To demonstrate sealing of injured myocytes with liposomes, fluorescein-labeled phospholipids were used in the production of various liposome preparations such as immunoliposomes and liposomes without antibody.

Liposome adherence to cardiomyocytes was assessed by fluorescence and confocal microscopy; electron dense immunoliposomes and liposomes were also prepared for ultrastructural characterization of the treated hypoxic cardiomyocytes by the following protocol:

Two million H9C2 myocytes were cultured in 25 ml culture flasks with DMEM and fetal calf serum at 37° C. in a humidified incubator with 5% CO_2 concentration. After the cells were stabilized at 37° C. incubation overnight, the cells were washed with phosphate buffer (pH 7.4) followed by addition of 3 ml fresh medium with or without liposomes (immuno or non-immuno) or antibody. To produce mild hypoxic injury, highly purified nitrogen was bubbled through the medium for 4 minutes without dislodging the cells from the flask bottom, the flasks were closed tightly, and incubated overnight at 37° C. for 24 hours. To induce severe injury, the stream of nitrogen was directed to dislodge almost all the cells from the bottom surface of the culture wells.

After an overnight hypoxic injury, only 14% of control myocytes were assessed to be viable by trypan blue exclusion criteria (FIG. 2). Most of the dead cells were recovered in the supernatant. The results showed that liposomes without antibody (42.3%) and nonspecific immunoliposomes provided some protection to the cells from hypoxic injury. This suggested that liposomes, which are a lipid bilayer, can nonspecifically stick to cell surfaces and fortuitously seal some of the cell membrane branches. However, in contrast to the moderate protection provided by liposomes without antibody and nonspecific immunoliposomes, immunoliposomes that were prepared according to the invention to specifically target myosin resulted in extraordinary salvage of the damaged cells from death. These immunoliposomes almost completely prevented cell death (96.2% viability as shown in FIG. 2) relative to 98% of normally grown cells. These results demonstrate that immunoliposome targeting of intracellular antigen produces selective targeting of the areas of myosin exposure and effectively seals the sites of membrane disruption. These severely hypoxic cells also showed cell viability when treated with immunoliposomes as determined by ^{3}H-thymidine uptake (FIG. 3). Immunoliposome treated cells had 83–85% viability relative to normoxic controls (100%). In contrast, cells treated with liposomes without antibody had only 19.4% viability, and control hypoxic cells had only 5.2% viability, as assessed by ^{3}H-thymidine uptake.

After severe hypoxia injury, myocytes treated with fluorescent immunoliposomes showed re-attachment of myocytes to the culture flasks, indicating cell viability and no loss of myocytes. Normally shaped mycocytes were usually seen as confluent, firmly adherent cultures with fluorescence visible on most of the cells. On the other hand, cultured myocytes treated with liposomes without antibody only demonstrated scattered cells in the culture flasks with fluorescence, or few clusters of cells with fluorescence. Most of these cells did not maintain normal cell morphology and shape, and did not appear healthy. The presence of immunoliposomes on the cell surfaces with retention of cell morphology was confirmed by confocal microscopy. The intensity of fluorescence or distribution of the immunoliposomes varied from myocyte to myocyte and depended on the severity of damage to the cells.

Referring to FIG. 4, the H9C2 rat cardiomyocytes were cultured in hypoxia for 24 hours with either fluorescent liposomes without antibody (left panel) or fluorescent antimyosin immunoliposomes (right panel). The immunoliposomes can be seen anchored (green colored dots) to the

hypoxia myocytes (seen as grey background) at the sites of myosin exposure through cell membrane breaches. The myocytes incubated with immunoliposomes were adherently attached to the bottoms of the flask, representing almost complete viability. In contrast, most of the myocytes cultured with plain liposomes in hypoxia conditions were dead and were recovered from the supernatant. The left panel demonstrates some ghost myocytes which are covered with some nonspecific plain liposomes (green hue). The results of this experiment clearly show that if a liposome is directed specifically against an intracellular cytoskeletal protein, it will seek the areas of cell membrane rupture precisely, will plug the breaches and will prevent loss of cell viability.

Referring to FIG. 5, liposomes were pseudocolored to enhance visualization of localization of immunoliposomes and liposomes without antibody. The attachment of immunoliposomes to hypoxic myocytes can be delineated. Liposomes without antibody, in the left panel, showed some nonspecific uptake on amorphic nonviable cells.

As shown in FIG. 6, another set of H9C2 cardiomyocytes incubated with antimyosin immunoliposomes demonstrated the same protective phenomenon. The left panel demonstrates the superimposition of the green fluorescence of immunoliposomes over the grey cardiomyocytes. The liposome distribution clearly demonstrates the areas of the cardiomyocytes that are normal and did not attract liposomes compared to the areas in the same cell that have dense localization of immunoliposomes. The differential liposomal distribution can be clearly visualized by pseudocolorization (right panel) of the same field of view. The experiment demonstrates the selectivity and specificity of the immunoliposomal localization since the injured areas (with anticipated myosin exposure) attracted the immunoliposomes and the normal areas with intact sarcolemma (and hence unexposed myosin) did not show any localization.

FIG. 7 shows another set of hypoxia H9C2 myocytes incubated with antimyosin liposomes for 5 days. As seen in the pseudocolored micrographs, immunoliposome treated cells were still viable at 5 days when cultured under hypoxia. Although the cell shapes have changed somewhat, the cell membrane is still clearly intact. The fluorescent lipids at this time were observed perinuclearly, appearing to be within the cytoplasm by confocal microscopy. No viable myocytes treated with liposomes without antibody were seen after 5 days.

Transmission electron microscopy demonstrated that fusion of the immunoliposomes had occurred with the membrane by demonstrating the attachment of immunoliposomes on the cell membranes as well as by showing intracellular delivery of patchy electron dense intraliposomal contents to otherwise unstained myocytes. Referring to FIG. 8, ultrastructural examination of the localization of silver grains containing immunoliposomes in hypoxia myocytes is shown. Confirmation of intracellular delivery of intraliposomal contents is evident in this electron micrograph. Antimyosin immunoliposomes used for this experiment contained silver grains in the liposomal core. After 24 hours of incubation with hypoxia cardiomyocytes, silver grains (dark black dots) were seen to be associated either with the membrane (arrow heads) above the myofilaments or observed towards the central part of the intracellular space (thin arrows). This experiment utilized silver grains as a model drug/genetic vector for the assessment of targeted intracellular delivery. The extent of silver grains delivered into this cell visible in this thin section suggests that immunoliposomes directed against a cytoskeletal protein may offer a highly efficient tool for gene delivery into cells. The methodology will have an additional advantage of salvaging the stressed cells by plugging the cell membrane breaches.

In vivo Determination of Efficacy of Immunoliposome Sealing

In order to test for immunoliposome efficacy in vivo, e.g., in reduction of myocardial necrosis, a canine myocardial infarct model is used, as described in Khaw et al., Circulation 60:1527 (1979) and Narula et al., Jour. Nuclear Med. 35:1076 (1994), both of which are hereby incorporated by reference. Antimyosin imaging, histochemical and pathological assessment of the infarct sized is performed to compare the efficacy of treatment to reduce infarct size.

1. Canine Model

Mongrel dogs of both sexes weighing 20 to 30 kg are anesthetized with intravenous sodium pentobarbital (30 mg/kg) and ventilated with a Harvard positive-pressure respirator (Harvard Apparatus, South Natick, Mass.). The right femoral artery and vein are isolated and catheterized to facilitate monitoring of arterial pressures and the administration of medication. Arterial pressure and a multilead electrocardiogram are continuously monitored. A left thoracotomy is performed, and the heart is suspended in the pericardial cradle. Proximal and midportions of left anterior descending coronary artery (LAD) are isolated, and a surgical monofilament snare enclosed in polyethylene tubing is placed immediately distal to the first diagonal branch. A small branch just distal to the site of occlusion is cannulated with a 22-gauge catheter for the administration of antimyosin immunoliposomes into the LAD. The LAD is occluded by tightening the snare, and a prophylactic lidocaine infusion of 0.1 mg/kg is maintained for 1 hour. At 1.5 hour, the LAD territory is reperfused by removal of the snare. At the time of reperfusion, approximately one-half the animals are randomly selected to receive a single dose of immunoliposomes dissolved in 10 ml of 0.9 percent saline through the LAD cannula at a rate of 1.1 ml/min. The remaining animals receive the same volume of intracoronary saline. The animals are killed at 24 hours or 7 days later by atrial injection of 20 to 40 mEq of potassium chloride for histolochemical and histological characterization of the infarcted myocardium. The heart is excised, the atria are removed, the ventricular mass is recorded in grams, and the ventricle is sliced into 1 cm thick slices.

Cell membrane sealing is assessed by survival/viability testing of tissue, using nitroblue tetrazolium staining. Alternatively, to demonstrate liposome sealing of injured cells, fluorescein-labeled phospholipids are used in the preparation of immunoliposomes, and detection of liposome adherence to cardiomyocytes is assessed by fluorescence microscopy and confocal microscopy, as taught herein. Light microscopy followed by transmission electromicroscopy are other methods of assessment of cell viability. Functional assessment of the hearts in vivo can be performed using gated blood pool imaging or echocardiography. Immunoliposome treated and saline treated animals will be compared.

2. Antimyosin Antibodies

A monoclonal antimyosin antibody was generated by hybridization of immune murine spleen cells with SP2/0A murine myeloma cells, purified by methods previously described (Khaw et al., Hybridoma 3:11–23, 1984, hereby incorporated by reference), and numbered 2G42D7 (as referred to herein). The bicyclic anhydride of diethylenetriamine pentaacetic acid (DTPA) is prepared by the method of Hnatowich et al. (Hnatowich et al., Science 220:613–615, 1983, hereby incorporated by reference) for coupling with

antimyosin-Fab. The molar ratio of DTPA to Fab was 1:1. Two approaches may be used to label antimyosin Fab. ^{111}In coupling to the DTPA and ^{123}I coupling directly to the protein using chloramine-T.

Indium Labeling.

Approximately 37 MBq (1 mCi) of ^{111}InCl is used to label 100 μg of DTPA-R11D10-Fab. To a 1-mCi aliquot (50 μl) of ^{111}InCl, an equal volume of 1M sodium citrate (pH 5.5) is added, followed by an aliquot of antimyosin-Fab. The reaction mixture is allowed to incubate at room temperature for 30 min. Antibody-bound ^{111}In is separated from free ^{111}In by SEPHADEX (Sigma Chemical, St. Louis, Mo.) G-25 column (10 ml) chromatography. The peak tubes in the void volume containing the radiolabled antibody are pooled and used within 1 hour of radiolabeling. An average of 80% of the initial antibody concentration is recovered in the peak tubes containing the radiolabeled antibody.

Iodination.

Radioiodination is accomplished by the chloramine-T method, as described by Hunter and Greenwood (Hunter et al., Nature 194:495–496, 1962) to label antimyosin Fab with ^{123}I. To a 100 μg aliquot of DTPA-coupled antimyosin Fab antibody in 0.1M phosphate buffer (pH 7.4), 37 MBq (1 mCi) of ^{123}I is added and mixed thoroughly. A 10-μl aliquot of chloramine T (26 mg/ml in 0.5M phosphate buffer, pH 7.4) is added followed by mixing for 2 min. Iodination is terminated by the addition of 25 μl of 0.1M methionine plus 0.1M cresol. The reaction mixture is then applied to a 10-ml SEPHADEX G-25 column to separate free and protein-bound radioiodine.

Antimyosin Antibody Imaging.

The following sets of images are recorded in each animal: in vivo during the experiment, of the whole heart ex vivo and as 1-cm ring slices cut perpendicular to the long axis of the left ventricle. Scintigraphic images are obtained using a gamma camera (Ohio-Nuclear 100, Solon, Ohio) equipped with a medium-energy collimator. The pulse height analyzer are set at center lines of 247 and 159 keV with 20% windows for ^{111}In and ^{123}I radioisotopes, respectively. Concurrent with antibody injections, sequential 1-min acquisition images are recorded for a total of 8 min. The excised hearts are imaged whole and as 1-cm thick slices for both isotopes. Background images are also collected at each isotope setting for identical acquisition time. The background corrected and peak-normalized sets of images are recorded on floppy disks and the experiment number, treatment and sequence of injection of ^{111}In-and ^{123}I-labeled antimyosin antibody are blinded. The infarct size is assessed twice individually by three observers. Semiautomatic planimetry is performed with a Technicare 560 computer (Technicare Corp., Solon, Ohio) to determine the infarct size as the number of pixels, as previously described (Khaw et al., J. Nucl. Med. 28:76–82, 1987). The pixel size is calibrated, and the absolute volume of infarcted tissue is determined (area×slice thickness). This value, multiplied by the specific gravity of myocardium (1.05), is used to determine the weight of the infarcted tissue in grams (Osuzega et al., Am. Heart J. 117:444–452, 1989). The percent infarct relative to the ventricular mass is then calculated for the statistical analysis.

Targeted Delivery of DNA

Several methods have been used for the delivery of DNA into cells, including poly-L-lysine conjugated lipids (Zhou et al., Biochim. Biophys. Acta. 1065:8–14, 1991), pH sensitive immunoliposomes (Gregoriadis, G., Liposome Technology, Vol I, II, III, CRC, 1993), and cationic liposomes (Felgner et al., Proc. Natl. Acad. Sci., USA. 84:7413–7417, 1987). However, the drawback with all of these methods is the insufficient uptake and expression of selected genes (DNA). Gene delivery and transfection can be enhanced by the use of hypoxic injury and immunoliposomes as targeting modality. Transmission electron micrographs showed that immunoliposomes can be used to deliver hundreds or more units of intraliposomal contents into an individual target cell (FIG. 8).

Dosage and Mode of Administration

Immunoliposomes administered according to the invention are administered intravenously, intraperitoneally or directly to the target tissue or organ, at a dosage that is appropriate for the amount of biological agent or genetic material that is encapsulated by the liposome. Immunoliposome dosage will therefore vary from about 5 mg/kg body weight to about 1 gm/kg body weight, and may be in the range of 100 mg–500 mg/kg body weight.

OTHER EMBODIMENTS

Other embodiments will be evident to those of skill in the art. It should be understood that the foregoing detailed description is provided for clarity only and is merely exemplary. The spirit and scope of the present invention are not limited thereto, being defined by the claims set forth below.

What is claimed is:

1. A method of inhibiting cell death in cardiac tissue, said method comprising

providing cardiac tissue comprising injured cardiomyocytes having intracellular myosin exposed to the exterior of said cardiomyocytes;

contacting said injured cardiomyocytes with a specific affinity reagent-liposome conjugate, said specific affinity reagent comprising an antibody specific for said intracellular myosin, for a time sufficient to allow said conjugate to adhere to said intracellular myosin; and

determining the viability of said cardiomyocytes.

2. The method of claim 1, wherein said conjugate comprises a biological agent.

3. The method of claim 2, wherein said biological agent is selected from the group consisting of fibroblastic growth factor-β, angiogenic factors, high energy substrates for the myocardium, antioxidants, cytokines and contrast agents.

* * * * *

Exhibit N

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Iodinated Contrast Agents for Brain Tumor Localization and Radiation Dose Enhancement

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Norman A, Iwamoto KS, Cochran ST. Iodinated contrast agents for brain tumor localization and radiation dose enhancement. Invest Radiol 1991;26:S120–S121.

IRRADIATING CELLS IN iodinated contrast media (CM) enhances the radiation dose absorbed from diagnostic x-rays.[1–5] We demonstrated the potential usefulness of this phenomenon for treating brain tumors by showing that the survival of rabbits after radiation therapy to a VX-2 brain tumor was increased when irradiation was preceded by an infusion of CM.[6] More recently we have demonstrated that the CT scanner which we use to localize brain tumors can also be used, with a modified collimator, to deliver radiation therapy.[7] The CM thus serves two functions: to help localize the tumor and to increase the absorbed radiation dose. We describe here the dose enhancement factor (DEF) that can be achieved with conventional administration of CM and discuss possible methods for increasing it.

Materials and Methods

We have been using a CTX, a GE 9800 CT scanner modified for therapy, operated at 140 KV to deliver multi-arc rotational therapy to spontaneous brain tumors in dogs.[7] The concentration of CM in the tumor can be estimated from the change in CT numbers over the tumor after infusion of CM. The corresponding DEF was determined by irradiating human peripheral blood lymphocytes in varying concentrations of CM at the isocenter of the CTX. Briefly, blood samples obtained from volunteers with informed consent were centrifuged, the buffy coat was suspended in culture medium to which various concentrations of CM were added, and the cell suspensions were placed in small plastic culture tubes. Following irradiation the cells were cultured for 24 hours, cytochalasin B was added, and the cells were cultured for another 48 hours. The cells were then fixed, air dried on slides and stained. The frequency of binucleate cells with micronuclei was then assayed under the microscope and used as a measure of the absorbed radiation dose.[8] The DEF was calculated as the ratio of the micronuclear frequencies in cells irradiated in CM to those irradiated without CM.

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Results

Figure 1 shows a plot of the DEF as a function of the iodine concentration in the lymphocyte medium during irradiation with the CTX at 140 kVp. It also shows the DEF calculated from the results of Dawson et al[5] obtained by irradiating Chinese hamster ovary (CHO) cells at 250 kVp and measuring survival by a colony assay. The two lines are the least square fits to the lymphocyte and CHO data, respectively. Two points obtained earlier by cytogenetic analysis of lymphocytes irradiated at 250 kVp[4] are also plotted. As can be seen the two points are in good agreement with the results of Dawson et al. As expected,[4] the DEF increases linearly with the concentration of iodine; and it is smaller for the higher kVp because of the decrease in the iodine photoelectric cross section with increasing kVp

Discussion

The therapeutic ratio, the ratio of radiation dose absorbed by a brain tumor versus that absorbed by the surrounding normal brain tissues, will increase with increasing CM in the tumor. One way to increase the DEF is to irradiate with x-rays photons near the 32 KeV absorption edge of iodine, but these will have poorer penetration through the skull and brain to the tumor than the 140 kVp x-rays. Another is to use a gadolinium CM; because it has a higher atomic number than iodine it may give a higher DEF at high kVp. Inspection of the absorption spectra suggest that at 140 kVp equal concentrations of gadolinium and iodine will give about the same DEF. A third way is to use a CM that is incorporated into the tumor cells; this will increase the radiation dose to the tumor by the action of photoelectrons and Auger electrons which do not reach the cell nucleus when the CM is excluded from the cells. One way to incorporate iodine into the tumor cell nucleus is to infuse iodinated deoxyuridine, a DNA precursor, over several

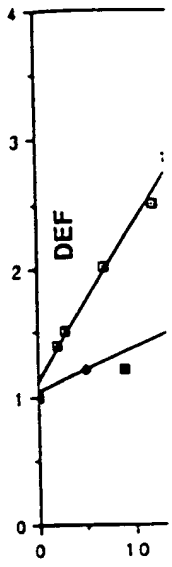


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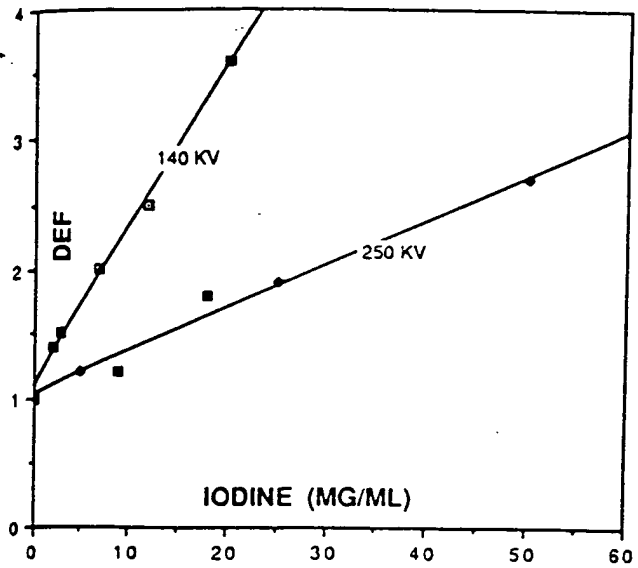


Fig. 1. Plot of the dose enhancement factor.

days[9]; but that is a difficult and expensive method as compared to injecting CM.

The straightforward way to increase the DEF is to increase the amount of iodinated CM that is injected into the patients. A primary bar to increasing the conventional CM dose is its toxicity. The development of new CM and of other strategies to reduce the toxicity will allow us to deliver larger iodine concentrations to the tumor and thus to increase the usefulness of iodine radiation dose enhancement therapy for brain tumors.

Conclusions

The use of iodinated CM and the CTX provides a simple and effective way to localize brain tumors and to deliver multi-arc rotational therapy to the tumors. The DEF from the iodine increases with the amount of CM that can be safely injected into the patient. The continued development of methods to decrease the toxicity of the iodinated CM promise to increase the effectiveness of iodine dose enhancement therapy for brain tumors.

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